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ANION AND METAL ION BINDING TO YEAST PHOSPHOGLYCERATE KINASE

by

Ⓒ JEAN ANNE WROBEL

A THESIS
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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled ANION AND METAL ION BINDING TO YEAST PHOSPHOGLYCERATE KINASE submitted by JEAN ANNE WROBEL in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE

to Ern Pearcey

who helped to clear my

thoughts at the end of the day

ABSTRACT

The binding of substrate and non-substrate anions, and metal ions, to yeast phosphoglycerate kinase was monitored spectrophotometrically using two probes directed toward the enzyme's single, non-essential thiol. The first system involved the enzyme labelled with the sulfhydryl reporter group 2-chloromercuri-4-nitrophenol. Titrations of the enzyme-mercurial complex with anions decreased the absorbance of the bound chromophore. Dissociation constants were determined from Scatchard or double-reciprocal plots of the data. A linear relationship was found between the charge of the anion and the negative logarithm of the dissociation constant for the anion. The average number of mol of bound anion was 1.06 per 47,000 g of labelled enzyme. Several lines of evidence suggested that titration of the catalytic center was not being monitored. Titration of the enzyme-mercurial complex with Mg^{2+}, Mn^{2+}, Co^{2+}, Ca^{2+}, or Cd^{2+} increased the absorbance of the bound chromophore. Dissociation constants for these metal ions were determined from Scatchard plots and ranged from 8 to 43 μM. A single mol of metal ion was bound per 47,000 g of labelled enzyme. In contrast, two Zn^{2+} binding sites were demonstrated and both appeared to be distinct from the other metal ion binding site. The dissociation constant for Zn^{2+} at its weaker binding site was $\sim$8 μM.

The second system used to monitor ligand binding involved the reaction between native phosphoglycerate kinase

and 5,5'-dithiobis(2-nitrobenzoic acid)(DTNB). The rate constant for the reaction decreased in the presence of each of the anions examined. A plot of the reciprocal of the rate constant *vs.* the concentration of anion was used to calculate the dissociation constant between anion and enzyme. Dissociation constants determined in this manner were in good agreement with those determined from titration of enzyme-mercurial complex. The presence of each of the divalent metal ions examined increased the rate constant for the reaction between enzyme and DTNB. Analysis of the data gave dissociation constants which agreed with those determined using the mercurial probe. Furthermore, the same two Zn^{2+} binding sites apparent from titrations of the enzyme-mercurial complex were also detected.

The anion, metal ion, and Zn^{2+} binding sites did not appear to interact in terms of binding affinities.

Modification of phosphoglycerate kinase with each of the thiol-directed probes increased the susceptibility of the enzyme to proteolytic inactivation by trypsin or yeast proteinase A compared to that observed with the native kinase. Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate revealed that limited proteolytic degradation had taken place during the incubation. The molecular weight of the major polypeptide end-products differed with the two endopeptidases. Succinate protected the native and mercurial-labelled kinase from inactivation by trypsin and proteinase A, respectively. Zn^{2+} increased the susceptibility of the native

kinase to inactivation by trypsin or proteinase A and decreased the susceptibility of the mercurial-labelled kinase to inactivation by proteinase A. The effects of succinate and Zn^{2+} were detected at concentrations compatible with occupancy of the anion and weaker Zn^{2+} binding sites, respectively, indicated from the spectrophotometric binding studies.

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LIST OF ABBREVIATIONS

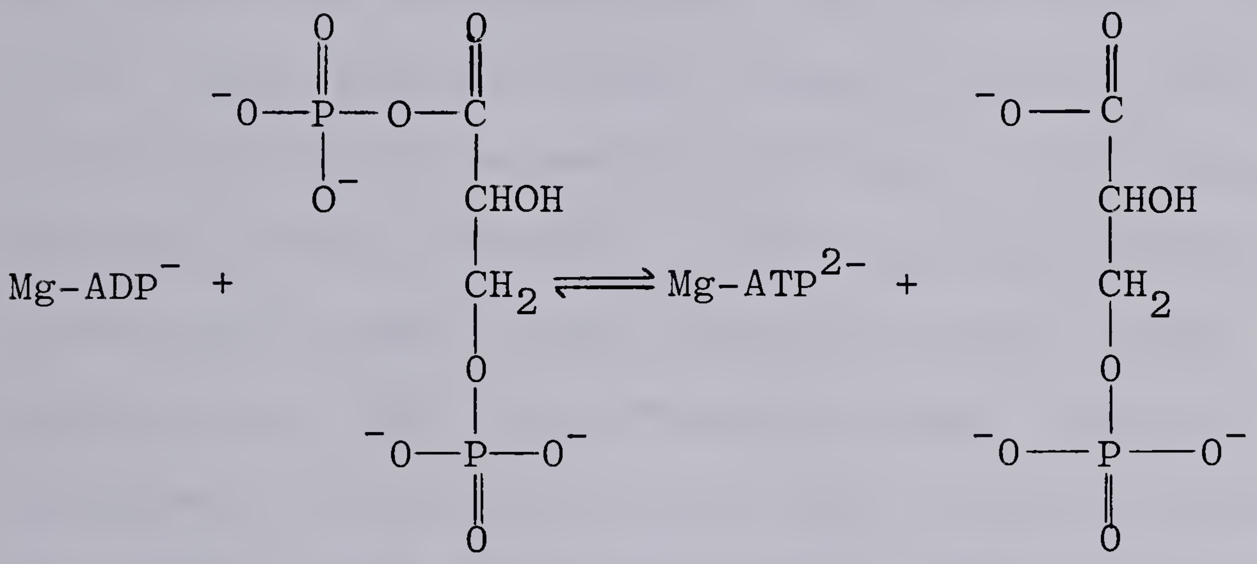
ADP	adenosine 5'-pyrophosphate
AMP	adenosine 5'-phosphate
ATP	adenosine 5'-triphosphate
CTP	cytidine 5'-triphosphate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
$E^{1\%}_{1\ \mathrm{cm}}$	absorbance of a 1% protein solution in a 1 cm pathlength cell
$E^{\mathrm{mM}}_{1\ \mathrm{cm}}$	absorbance of a 1 mM solution in a 1 cm pathlength cell
EPR	electron paramagnetic resonance
GTP	guanosine 5'-triphosphate
ITP	inosine 5'-triphosphate
K_i	inhibitor constant
K_m	Michaelis constant
mercurial	2-chloromercuri-4-nitrophenol
NMR	nuclear magnetic resonance
Tes	N-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid
TTP	thymidine 5'-triphosphate
UTP	uridine 5'-triphosphate

CHAPTER I

INTRODUCTION

A. REACTION CATALYZED

Phosphoglycerate kinase (EC 2.7.2.3) catalyzes the transfer of the "high energy" acyl-linkage phosphate group of 1,3-bisphosphoglycerate to ADP to yield ATP and 3-phosphoglycerate:



$$\text{Mg-ADP}^- + {}^-O\text{—}P(=O)(O^-)\text{—}O\text{—}C(=O)\text{—}CHOH\text{—}CH_2\text{—}O\text{—}P(=O)(O^-)\text{—}O^- \rightleftharpoons \text{Mg-ATP}^{2-} + {}^-O\text{—}C(=O)\text{—}CHOH\text{—}CH_2\text{—}O\text{—}P(=O)(O^-)\text{—}O^-$$

The reaction equilibrium favors production of ATP which is the direction of glycolysis. The equilibrium constant estimated at catalytic enzyme concentrations is ~ 3×10^4 (Bücher, 1947; Krietsch and Bücher, 1970) with the resulting ΔG^o equal to -17.5 kJ/mole (Scopes, 1973). Phosphoglycerate kinase from most sources participates in glycolysis but in photosynthetic plants the enzyme located within mitochondria takes part in carbon fixation. Because of the difficulty in preparation and instability of 1,3-bisphosphoglycerate (see for example Negelein, 1957), enzyme activity is usually determined in the reverse, or ATP-utilizing, direction. Most often a

glyceraldehyde 3-phosphate dehydrogenase-linked assay near pH 7.5 is used (see for example Krietsch and Bücher, 1970).

B. PURIFICATION

Purification of phosphoglycerate kinase from yeast includes the following steps: ammonia cytolysis, ammonium sulfate fractionation, and ion exchange and gel filtration chromatography (see for example Scopes, 1971). When assayed in the reverse direction such preparations have a specific activity of ~850 U/mg (Stinson, 1974). Recently, affinity chromatography (Cavell and Scopes, 1976; Pegorara and Lee, 1978; Kuntz et al, 1978) and affinity elution techniques (Cottreau et al, 1976; Scopes, 1977 a and b; Stewart and Scopes, 1978; Fifis and Scopes, 1978) have been described, and homogeneous preparation of phosphoglycerate kinase from a variety of sources have been claimed with as few as two purification steps.

C. INTRACELLULAR LOCATION

Early reports suggested that the soluble phosphotriose-glycerate phosphate group of glycolytic enzymes formed a multi-enzyme complex but this has not been verified experimentally in preparations of chicken breast muscle (Melnick and Hultin, 1973). Mowbray and Moses (1976) have demonstrated a glycolytic complex from *E. coli*,

and Fossel and Solomon (1978) claimed that a similar complex which included phosphoglycerate kinase activity could be isolated from broken human erythrocyte ghosts.

Despite reports of the association between the glycolytic enzymes and muscle proteins (see for example Clarke and Masters, 1975) evidence that this is of biological importance during muscle contraction has not been presented.

Based on the metabolic properties of red cell ghosts, Arese et al (1974; 1977) have presented evidence for membrane-associated phosphoglycerate kinase activity. Direct studies (Duchon and Collier, 1971; Schrier et al, 1975; Tillman et al, 1975; De and Kirtley, 1977) have indicated that the proportion (1 to ~13%) and tightness of phosphoglycerate kinase binding to red cell membranes depends on the conditions of isolation.

Membrane-associated glyceraldehyde 3-phosphate dehydrogenase and phosphoglycerate kinase have been implicated in cellular uptake of inorganic phosphate (Barlett, 1958; Ronquist, 1967; Niehaus and Hammerstedt, 1976) and in providing a local membrane ATP pool (Arese et al, 1977) for active Na^+/K^+ transport (Parker and Hoffman, 1967; Segel et al, 1975; Proverbio and Hoffman, 1977). However, the latter function has recently been disputed (Chillar and Beutler, 1976; De and Kirtley, 1977).

D. MOLECULAR PROPERTIES

1. Molecular Weight

A single band on polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate is a consistent finding with enzyme purified from a variety of sources, and the molecular weight determined by this and other physical methods falls within the range of 45,000 to 48,000 (see for example, Stewart and Scopes, 1978; Fifis and Scopes, 1978). Spragg and co-workers (1976) used the ultracentrifuge equilibrium method and determined a molecular weight of 40,030 for the yeast enzyme at concentrations less than 0.1 g/100 cm^3. These authors pointed out that their value was closest to that for a protein containing 357 residues as indicated from the X-ray studies of Bryant et al (1974). Furthermore, they presented evidence for monomer-dimer association and suggested that such associations could account for overestimation of molecular weights determined at higher enzyme concentrations. Gupta and Rothstein (1976 a and b) claimed that a heat stable factor from the nematode *Turbatrix aceti* caused, at low ionic strength, aggregation of partially purified phosphoglycerate kinase from the same source. These workers further suggested that, depending on the method of purification, similar aggregation could be demonstrated with the partially purified rat liver or yeast enzymes.

2. Primary Structure

Amino acid analysis of the enzyme from photosynthetic,

gluconeogenic, or glycolytic sources indicates a protein of 420 to 424 residues with remarkable consistency of amino acid composition despite a diversity of origin (see for example Scopes, 1973). However, a notable difference is the single cysteinyl residue of the yeast, bacterial, and 'photosynthetic' enzymes compared to the usual 5 to 10 thiol groups present in the kinase from most other sources (Cavell and Scopes, 1976; Stewart and Scopes, 1978, Fifis and Scopes, 1978). As with most of the glycolytic enzymes (Jörnvall, 1975) the N-terminus of phosphoglycerate kinase from yeast (Fattoum et al, 1978) and human red cells (Yoshida, 1972) is acetylated; the blocked N-terminus of the rabbit muscle enzyme has not yet been identified (Krietsch and Bücher, 1970). The C-terminal residues of yeast, rabbit muscle and human red cell phosphoglycerate kinase are lysine (Markland et al, 1975; Fattoum et al, 1978), valine (Krietsch and Bücher, 1970), and isoleucine (Yoshida and Watanabe, 1972), respectively.

3. Secondary and Tertiary Structure

Electron density maps at 5- and 3.5-Å resolution indicate that yeast phosphoglycerate kinase is a monomeric enzyme, prolate ellipsoid in shape with dimensions of 85 x 55 x 55 Å. The molecule contains two lobes of similar size connected by a central waist region and each lobe contains a core β-pleated sheet structure surrounded by helical segments. The waist region contains two helices and since the density in this area is weak considerable flexibility could occur across it in solution. Although

the mercury and gold heavy atom sites are 6 Å apart this was attributed to secondary interactions and it was concluded that both reagents bound to the enzyme's single thiol situated on lobe B. The substrate Mg-ADP binds in the cleft region of lobe A, and is some 30 Å removed from the heavy atom binding site (Wendell et al, 1972, Bryant et al, 1974). Attempts thus far to localize the sugar substrate binding site of the crystallized enzyme have been unsuccessful, and this has been attributed to competion by sulfate (Blake and Evans, 1974; Watson et al, 1977).

Similar structural results were obtained from X-ray crystallographic studies of the horse muscle enzyme at 6 Å (Blake et al, 1972) and 3 Å (Blake and Evans, 1974) of resolution. In addition, Mg-ATP and Mg-ADP binding studies indicated that phosphate moieties were extended into the space between the lobes such that the γ-phosphate of ATP was in contact with a residue side chain on the opposite lobe, and this prompted the authors to suggest that both lobes were involved in the catalytic reaction (Blake and Evans, 1974). Such extention of the phosphate chains has not been demonstrated in NMR substrate binding studies, and has been attributed to sulfate interference with the X-ray studies since this anion has been shown to bind at the β- and γ-phosphoryl binding sites(Tanswell et al, 1976).

E. ISOENZYMES AND GENETICS

Early claims of differences in electrophoretic mobilities or isoelectric points of the enzyme prepared from different sources (Krietsch and Bücher, 1970; Yoshida et al, 1972; Bojanovski et al, 1974; Heinz et al, 1974) are complicated by the ability of phosphoglycerate kinase to bind a variety of anionic buffers (see for example Scopes, 1973). Demonstration of variants of the enzyme has also been shown to depend on the buffer system (Yoshida et al, 1972; Cam and Cooper, 1978). Furthermore, the mobility of the enzyme from most sources is influenced by the presence of reducing agents. For example, in the absence of such agents the enzyme purified from rabbit muscle will give multiple bands on electrophoresis despite having migrated as a single band in the crude extract (Scopes, 1969).

The structural gene coding for the major phosphoglycerate kinase component in most tissues (A isoenzyme) is located on the X-chromosome in man (see for example Shows and Brown, 1975). Expression of enzyme activity is under X-chromosome control in other mammals (see for example Chapman and Shows, 1976) and marsupials (Cooper et al, 1971). A minor electrophoretically distinct form of phosphoglycerate kinase was first observed in marsupial hemolysates; since genetic analysis indicated an autosomal co-dominant mode of inheritance, this form was classified as an isoenzyme and designated as phosphoglycerate kinase B (Cooper et al, 1971). Subsequent studies indicated that the B isoenzyme was the

major component present in mammalian testicular tissue (Vande Berg et al, 1973), and Vande Berg and co-workers (1976) demonstrated that in mouse testes, the germ cell line was the source of the B isoenzyme.

Similarities in the kinetic and molecular properties of the A and B isoenzymes purified from ram testes (Stewart and Scopes, 1978), or mouse muscle and testicular tissue (Pegoraro and Lee, 1978) suggests that the gene coding for phosphoglycerate kinase B arose from duplication and shift of the X-chromosome structural gene coding for phosphoglycerate kinase A (Masters and Holmes, 1975).

Polymorphism of the A isoenzyme has been documented in primates (Ritter and Schmidt, 1974) and in man (Chen and Giblett, 1972; Yoshida et al, 1972) and rare human variants have been reported (see for example Kahn et al, 1976; Krietsch et al, 1977). Polymorphism of the human B isoenzyme has not yet been demonstrated (Chen et al, 1976).

Arvidsson and co-workers (1976) reported that autolysis of yeast in the absence of toluene yields a major and minor active component on electrophoresis; since analysis of primary and secondary structure, and specific activity determinations revealed only minor differences, the two forms were termed charge isomers, the minor component likely arising from the major component through cleavage of an amide bond.

Larsson-Raźnikiewicz (1970a), reported that commercially available preparations of yeast phosphoglycerate kinase

contained three active components on electrophoresis; the major and one of the minor components showed similar amino acid compositions, molecular weights, and sedimentation velocities, and the author reasoned that amide formation accounted for the differences in mobility. This hypothesis is consistent with recent isoelectric focusing studies by Stinson (1977) which indicated that a minor, more positive component which is fully active appears during storage of the purified yeast enzyme in 4 M ammonium sulfate.

F. KINETICS

1. Substrate Specificity

a) Nucleotide Substrates

The nucleotide specificity of phosphoglycerate kinase from most sources is such that the purine nucleotides ATP, GTP, and ITP can serve as the phosphate donors but differences in K_m and V_{max} values are apparent; ATP is the preferred substrate. Activity is absent or minimal when the pyrimidine nucleotides UTP, CTP, and TTP are substituted (see for example Krietsch and Bücher, 1970). Phosphoglycerate kinase from yeast and *Bacillus stearothermophilus* has been successfully inactivated by the reactive AMP analog N^6-(*p*-bromo acetoaminobenzyl)-AMP (Suzuki et al, 1977). According to Reeves and South (1974) the enzyme partially purified from *Entamoeba histolytica* showed greater specificity towards the guanine nucleotides. Phosphoglycerate kinase isolated from the nematode *Turbatrix aceti* showed activity with

ATP but not with ITP or GTP (Gupta and Rothstein, 1976b), and a recent report (Krietsch and Kuntz, 1978) indicated that the enzyme prepared from the blue alga *Spirulina platensis* is also specific for ATP.

From the early kinetic studies by Larsson-Raznikiewicz (Larsson-Raźnikiewicz and Malmström, 1961; Larsson-Raźnikiewicz, 1964) it was concluded that the metal ion complexes of ATP and ADP are the active substrate forms (see this chapter, section F.3). This has been substantiated by the proton relaxation rate and EPR substrate binding studies of Chapman et al (1974; 1977) which showed that although the free nucleotides do bind to the active center, the presence of a divalent metal ion such as Mn^{2+} decreases the dissociation constants.

b) Sugar Substrates

Orr and Knowles (1974) have determined the K_m values of yeast phosphoglycerate kinse for 3-phosphoglycerate and its phosphonate analog 2-hydroxy-4-phosphono-DL-butyrate. These authors argued that the dianionic state of the C-3 phosphoryl group is a major determinant of the affinity of 3-phosphoglycerate binding. It is interesting that pH profiles for the enzyme prepared from yeast or rabbit skeletal muscle show an optimum from pH 6 to 9.2 (see for example Krietsch and Bűcher, 1970), and this suggests that the final ionization constant of 3-phosphoglycerate (pK_a 6.25 Larsson-Raźnikiewicz, 1972) could contribute to the effect of pH on catalytic activity. Earlier kinetic studies had indicated that Mg-3-phospho-

glycerate was an inactive substrate form (Larsson-Raźnikiewicz, 1967).

According to the spectrophotometric studies of Roustan et al (1973) the sugar substrate binding site was highly specific for 3-phosphoglycerate since DL-1-glycerophosphate, 2-phosphoglycerate, and 2,3-bisphosphoglycerate did not bind. Chapman et al (1977) showed formation of an enzyme-Mn-ADP-3-phosphoglycerate complex but binding of 2-phosphoglycerate to the enzyme-Mn-ADP complex could not be demonostrated.

2. Substrate Binding Studies

Non-linear Lineweaver-Burk plots (concave downwards) for Mg-ATP (Larsson-Raźnikiewicz, 1967; Yoshida and Watanabe, 1972; Cavell and Scopes, 1976), 3-phosphoglycerate (Larsson-Raźnikiewicz, 1967; Krietsch and Bücher, 1970), Mg-ADP, and 1,3-bisphosphoglycerate (Ali and Brownstone, 1976) are a consistent finding with phosphoglycerate kinase from a variety of sources, and the presence of two sets of substrate binding sites has been proposed to account for this. Using the technique of equilibrium dialysis, Larsson-Raźnikiewicz (1973) reported the first direct evidence that at pH 7.8 two molecules each of free ATP or 3-phosphoglycerate bound per molecule of enzyme, although she could not clearly distinguish between the relative affinities of the two sites. Roustan et al (1973) also showed that the adenine nucleotides bound to the enzyme in the absence of metal ions at pH 7.2 but a single binding site was indicated. In a kinetic study of the yeast enzyme

designed to verify the use of the Ln-ATP complexes as active site probes for NMR studies, Tanswell et al (1974) observed that when gel filtration binding studies were carried out at pH 7.8, two Mg-ATP binding sites were indicated; however, at pH 6.3 NMR binding studies suggested one Mg-ATP, Ln-ATP, or Mg-ADP binding site. A single metal ion-nucleotide binding site is consistently found when X-ray crystallographic techniques are employed (Blake et al, 1972; Bryant et al, 1974; Blake and Evans, 1974) or with NMR techniques in the presence of 15 mM sulfate (Tanswell et al, 1976). From the above reports, it is apparent that whether one or two (metal ion-)nucleotide substrate binding sites are demonstrated depends on the methodology and experimental conditions.

A sugar substrate binding site on the crystallized enzyme has not yet been demonstrated (Blake and Evans, 1974; Watson et al, 1977).

3. Metal Ion Specificity

Kinetic studies indicated that the true nucleotide substrates are the metal ion complexes (see this chapter, section F.1.(a)). Substrate binding studies showed that Mn-ADP and, to a lesser extent, Mn-ATP bound more tightly to the yeast enzyme that did the respective free nucleotides (Chapman et al, 1974; 1977), and an $E\begin{smallmatrix} \diagup & M \\ & | \\ \diagdown & S \end{smallmatrix}$ complex of Mn-ADP with the horse muscle enzyme has been proposed (Blake and Evans, 1974). According to Larsson-Raźnikiewicz (1970b) the Mg^{2+}, Mn^{2+}, Ca^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}, or Ni^{2+} complexes of ATP are active substrate forms while activity cannot be

demonstrated with Be^{2+} or Fe^{2+}. The Mg^{2+} complex of ATP is likely the active substrate form of the yeast enzyme under physiological conditions (Malmström and Larsson-Raźnikiewcz, 1962).

Mg^{2+} is a competitive inhibitor with respect to Mg-ATP (Larsson-Raźikiewicz, 1964) at low free metal ion concentrations. At high concentrations of Mg^{2+} and Mn^{2+} inhibition is uncompetitive with Mg-ATP ($K_i \sim 3$ mM) and Mn-ATP ($K_i > 1.0$ mM), respectively (Larsson-Raźnikiewicz, 1970b), and at high Mg^{2+} to ATP ratios non-linear Lineweaver-Burk plots for 3-phosphoglycerate and Mg-ATP are noted (see for example Larsson-Raźnikiewicz, 1967). In the absence of substrates, Mn^{2+} binds to the enzyme with a dissociation constant of ~1.0 mM (Chapman et al, 1974). Direct binding of Mn^{2+} by the yeast enzyme was not indicated in the NMR studies of Tanswell et al (1976) but arguments presented by Chapman et al (1977) attributed this negative finding to the limitations of the monitoring conditions. Free Co^{2+} appears to activate the enzyme (Larsson-Raźnikiewicz, 1970b) and a dissociation constant of ~0.36 mM was estimated by equilibrium dialysis (Larsson-Raźnikiewicz, 1970b). Both Zn^{2+} and Cd^{2+} are strong inhibitors of the yeast enzyme, and the uncompetitive inhibitor constant for Zn^{2+} with respect to Zn-ATP is ~0.02 mM (Larsson-Raźnikiewicz, 1970b). Free Zn^{2+} and Cd^{2+} also strongly inhibit phosphoglycerate kinase purified from the silver beet (Cavell and Scopes, 1976).

4. Essential Residues

The following residues have been implicated in substrate binding to yeast phosphoglycerate kinase; tyrosyl (Markland et al, 1975; Meyer and Westhead, 1976; Hjelmgren, Arvidsson, and Larsson-Raźnikiewicz, 1976; Roustan et al, 1976), arginyl (Hjelmgren, Strid, and Arvidsson, 1976; Rogers and Weber, 1977; Phillips et al, 1978), and lysyl (Markland et al, 1975). Bacharach et al (1977) have isolated a nitrotyrosine-containing fragment following chymotryptic treatment of the nitrated, inactive enzyme. These authors argued that this peptide (lys-NO_2tyr-phe-phe-lys) fits well into the electron density map of the yeast enzyme and is located on the ATP-binding lobe (see this chapter, section D.2) but on the opposite side to the nucleotide binding site.

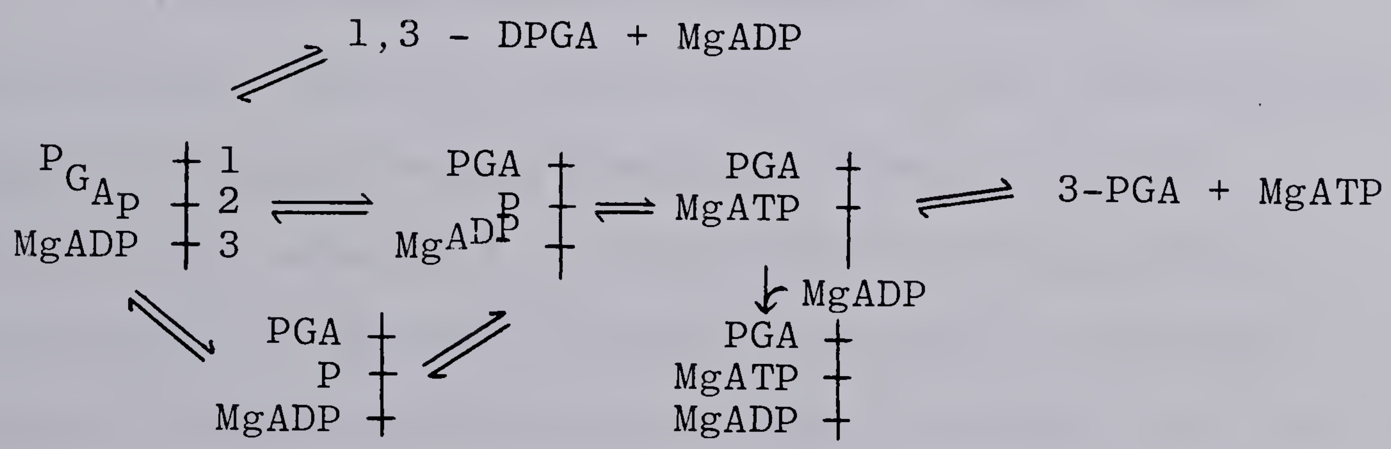
Brevet et al (1973) have claimed that carbodiimide modification of one carboxyl group resulted in enzyme inactivation and although 3-phosphoglycerate or each of the nucleotide substrates bound to the modified enzyme, the partial isotope exchange reaction between ADP and ATP was no longer demonstrable (but see this chapter, section F.5). Implication of an essential carboxyl group was also proposed by Brake and Weber (1974), and Bacharach et al (1977) have inactivated the enzyme by modification of a single carboxyl group and isolated a peptide which contained the modified residue.

According to Brevet et al (1973) modification of four histidyl residues did not influence catalytic activity. Markland et al (1975) reported that the three methionine

residues of the yeast enzyme are not essential for activity. However, three methionine and two tryptophan residues have been localized in the adenine binding domain of the active center (Watson et al, 1977). Modification of the single thiol of yeast phosphoglycerate kinase does not influence catalytic activity (Rao and Oesper, 1961; Krietsch and Bücher, 1970; Arvidsson and Larsson-Raźnikiewicz, 1973, Stinson, 1974; Markland et al, 1975).

5. Reaction Mechanisms

Based on kinetic studies of the yeast enzyme (Bücher, 1947, Larsson-Raźnikiewicz and Malmström, 1961; Larsson-Raźnikiewicz, 1964; 1967; Larsson-Raźnikiewicz and Arvidsson, 1971; Janson and Cleland, 1974) a rapid equilibrium, random mechanism with no mutual interference of substrate binding was proposed. The reaction scheme proposed by Bücher (1947) and modified by Scopes (1973) is shown below. 1,3-Bisphosphoglycerate is abbreviated as PGAP and PGA indicates 3-phosphoglycerate.



According to arguments presented by Malmström and Larsson-Raźnikiewicz (1962), Bücher's results were also compatible with a single ADP/ATP binding site, and more recent studies

support this idea (see this chapter, section F.2). In keeping with the proposed mechanism, substrate binding studies indicate that the nucleotide substrates and 3-phosphoglycerate will bind independently (see this chapter, section F.2).

Note that in the above reaction scheme provision is made for the formation of a phosphoenzyme intermediate. Claims to demonstrate a phosphoenzyme intermediate of phosphoglycerate kinase have been made (Walsh and Spector, 1971; Roustan et al, 1973) but can be attributed to contamination of enzyme preparations with other kinases (see for example Larsson-Raźnikiewicz and Schierbeck, 1974) or 3-phosphoglycerate introduced during the purification steps (see for example Johnson et al, 1975; 1976). These are two explanations as to why the reported partial exchange reaction between ADP and ATP was slower than the reaction in the presence of 3-phosphoglycerate and why variations in the proportion of phosphorylated enzyme were noted.

It has been recently suggested (Scopes, 1978b) from substrate binding studies that purified yeast phosphoglycerate kinase demonstrates trace ATPase activity. However, this also could be attributed to small amounts of contaminating 3-phosphoglycerate which were introduced in an affinity chromatographic step during the enzyme purification, and remained bound despite "extensive" dialysis.

G. AIMS OF THE PROJECT

The purpose of this project is to monitor the affinity and stoichiometry of binding of substrates and other anions, and metal ions to phosphoglycerate kinase purified from yeast. The experimental approach will involve the use of two thiol-directed probes with properties such that ligand binding can be monitored spectrophotometrically. The effect of these probes, anions, and metal ions on the susceptibility of yeast phosphoglycerate kinase to inactivation by trypsin and yeast proteinase A are also examined.

CHAPTER II

GENERAL MATERIALS AND METHODS

A. GENERAL MATERIALS

Yeast phosphoglycerate kinase was purchased from Boehringer Mannheim GmbH (Mannheim, F.R.G.) or Sigma Chemical Co. (St. Louis, Mo.), or prepared as previously reported (Stinson, 1974). Enzyme purity was assessed by isoelectric focusing (see this chapter, section B.3) and polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate (see this chapter, section B.9). Specific activities (see this chapter, sections B.1 and B.2) of approximately 700 U per mg were determined throughout the course of this work and are consistent with values previously obtained in this laboratory. Prior to use the enzyme was extensively dialyzed against the appropriate buffer to remove contaminating ions. Yeast glyceraldehyde 3-phosphate dehydrogenase was a product of Sigma Chemical Co. (St. Louis, Mo.) and prior to use was extensively dialyzed against 50 mM triethanolamine, pH 7.5, which contained 10 mM β-mercaptoethanol.

Bovine pancreatic trypsin (EC 3.4.4.4) and yeast proteinase A (EC 3.4.22.9) were purchased from Sigma Chemical Co. (St. Louis, Mo.). Lyophilized trypsin, with or without diphenyl carbamyl chloride, was prepared at a concentration of 5 mg per ml in 1 mM HCl, or 215 μM based on a molecular weight of 23,281 (Barman, 1969). A further dilution of this stock was made into 1 mM HCl to give a final concentration

of 0.42 μM. Lyophilized proteinase A contained 50% (w/w) citrate and was reconstituted with water to give a 5 mg protein per ml of solution. A concentration of 83 μM was calculated based on a molecular weight of 60,000 (Hata et al, 1967). All trypsin and proteinase A solutions were stored at 4^o.

2-Chloromercuri-4-nitrophenol was the generous gift of Dr. D.R. Trentham (University of Pennsylvania, USA). Triethanolamine, Tes, and DTNB were purchased from Sigma Chemical Co. (St. Louis, Mo.). Sodium hydroxide and hydrochloric acid were products of Fisher Scientific (Fairlawn, N.J.).

Acrylamide, bisacrylamide, sodium dodecyl sulfate, N,N,N',N'-tetramethylethylenediamine, all of high purity, and the subunit molecular weight markers were obtained from Bio-Rad Laboratories (Richmond, Calif.). Ammonium persulfate, glycerol, isopropanol, acetic acid, ethanol, and trichloroacetic acid were products of Fisher Scientific (Fairlawn, N.J.). Bromphenol blue was obtained from BDH Chemicals (Toronto, Canada) and Coomassie brilliant due R-250 was a product of Sigma Chemical Co. (St. Louis, Mo.). Carrier ampholytes (pH 7to 9) were purchased from LKB-Produkter A.B. (Bromma, Sweden).

All water was deionized and double distilled, with glass distillation as the final procedure.

Most spectrophotometric procedures were carried out with a Beckman Acta CIII spectrophotometer at 30^o. Some assays were monitored in a Pye Unicam SP6-300 spectrophotomer

at 30° coupled to a Fisher Recordall 5000 recorder.

B. GENERAL METHODS

1. Measurement of Phosphoglycerate Kinase Activity

Phosphoglycerate kinase was assayed in the reverse direction (see Chapter I, section A) as described by Krietsch and Bücher (1970) with minor changes. The assay buffer was 50 mM triethanolamine adjusted to a final pH of 7.5 with sodium hydroxide. The reaction was monitored at 340 nm. One unit of activity (U) is defined as that amount of enzyme which will catalyze the phosphorylation of 1 μmole of 3-phosphoglycerate per minute.

2. Protein Determination

Phosphoglycerate kinase protein concentration was determined using $E^{1\%}_{1cm}$ 4.9 at 280 nm (Krietsch and Bücher, 1970) and a molecular weight of 47,000 (Scopes, 1973). The concentration of the DTNB-modified enzyme was determined by calculating the concentration of the released 3-carboxylato-4-nitrothiophenolate anion (E^{mM}_{1cm} 14.1 at 412 nm Collier, 1973) in the presence of 10 mM β-mercaptoethanol buffered at pH 7.5 with 50 mM triethanolamine. Excess DTNB and nitrothiophenolate anion generated during the modification reaction had been removed by dialysis against 50 mM triethanolamine (pH 7.5) prior to quantitation of the modified protein.

3. Isolectric Focusing of Phosphoglycerate Kinase

Isolectric focusing of yeast phosphoglycerate kinase was as previously described (Stinson, 1977). Proteins were stained by the Coomassie blue method of Allen et al (1974).

4. Labelling of Phosphoglycerate Kinase with Mercurial and Titrations of the Enzyme-Mercurial Complex

Stock solutions of 2-chloromercuri-4-nitrophenol were prepared in 0.1 M NaOH, quantitated as previously described (Stinson, 1974), and stored at 4°. To label the enzyme with the mercurial approximately 10 to 20 μM of the enzyme was incubated with a 5% molar excess of mercurial for 3 to 5 min at 30° in 5.0 mM Tes, pH 8.0. The final volume was 1.0 ml and less than 4 μl of the mercurial were added for most experiments.

Additions of ligand were made with a Hamilton syringe equipped with a repeating dispensor (Hamilton Syringe Co., Cambridge, Mass.). The total volume of ligand solution added was usually not more than 20 μl and concentrations of reactants and absorbance values were corrected for this dilution. The change in absorbance at 410 nm of the enzyme-mercurial complex with each addition of ligand was monitored. Control titrations were done in the absence of enzyme but in the presence of mercurial bound to thioglycollic acid or β-mercaptoethanol.

5. Modification of Phosphoglycerate Kinase with DTNB in the Absence and Presence of Ligands

DTNB (39.6 mg) was added to approximately 8 ml of 5.0 mM Tes buffer, pH 8.0. This solution was constantly stirred and brought to a final pH of 8.0 with slow addition of approximately 2 ml of 0.1 N NaOH. The clear, pale yellow solution was brought to a volume of 10.0 ml with buffer to give a final concentration of 10.0 mM and was stored at 4^{o}.

For most experiments 5.0 or 10.0 μM of phosphoglycerate kinase was reacted with 0.2 or 1.0 mM DTNB and the release of the nitrothiophenolate anion was monitored at 412 nm. The concentration of anion released was calculated as described above (see this chapter, section B.2). The reaction was started with the addition of enzyme and blanks contained all components except the enzyme. Pseudo-first-order rate constants were calculated by the endpoint or difference method as described by Gutfreund (1965).

6. Wavelength Scans

Wavelength scans (520 to 260 nm) of 0.05 mM DTNB and 0.05 mM of the nitrothiophenolate anion were done in the absence and presence of metal ions. The nitrothiophenolate anion was released from DTNB following addition of freshly prepared β-mercaptoethanol to a final concentration of 0.2 mM. The effect of most metal ions on the spectra of DTNB or the nitrothiophenolate anion were determined immediately following addition of the metal ion or after up to 40 min incubation at 30^{o}. Mn^{2+}, Co^{2+}, Mg^{2+},

Ca^{2+}, Cd^{2+}, Cu^{2+} or Ni^{2+} were added to a final concentration of 50 and 250 μM while the effect of Zn^{2+} was assessed at 5 and 25 μM, and that of Cu^{1+} at 0.5 mM.

7. Proteolytic Inactivation of Phosphoglycerate Kinase

Phosphoglycerate kinase was modified with DTNB as described in this chapter (section B.5) then quantitated as outlined in this chapter (section B.2). Modification of the kinase with mercurial was according to the standard protocol (see this chapter, section B.4) but was carried out in 50 mM triethanolamine, pH 7.5. Native or modified phosphoglycerate kinase (2.0 μM) in 50 mM triethanolamine (pH 7.5) was pre-incubated at 30° for approximately 5 min. Trypsin was added to a final concentration of 2.9 μM when phosphoglycerate kinase was in the native form or to 0.004 μM when the kinase was modified with mercurial or DTNB. Proteinase A was added to a final concentration of 0.22 μM. All incubations were at 30°. Aliquots were taken prior to the addition of the endopeptidase and at timed intervals thereafter, and assayed as described (see this chapter, section B.1). Percent inactivation was calculated relative to the activity determined prior to the addition of endopeptidase. When polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate was simultaneously run, a second aliquot was taken after an additional min of incubation and immediately prepared for electrophoresis.

8. Hydrolysis of Benzoyl Arginine *p*-Nitroanilide by Trypsin

The rate of hydrolysis of benzoyl arginine

p-nitroanilide by trypsin was determined according to the method of Erlanger et al (1961) with the exceptions that the substrate was dissolved in water and the final concentration of buffer was 50 mM triethanolamine, pH 7.5.

9. Polyacrylamide-Gel Electrophoresis in the Presence of Sodium Dodecyl Sulfate

Polyacrylamide-gel (10%) electrophoresis in the presence of 0.1% sodium dodecyl sulfate was carried out as described by Weber and Osborn (1969). Proteins were stained with Coomassie blue according to the method of Fairbanks et al (1971). Molecular weights were determined from a plot of the R_f values of the marker proteins *vs.* the log of their molecular weights. The subunit protein markers were: phosphorylase *b*, 94,000; bovine serum albumin, 68,000; ovalbumin, 43,000; carbonic anhydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,300.

CHAPTER III

THE EFFECTS OF THE SULFHYDRYL REAGENTS ON SOME PROPERTIES OF PHOSPHOGLYCERATE KINASE

A. INTRODUCTION

1. General

Since the proposed ligand binding studies include the monitoring of substrate binding, a structural probe directed toward a non-essential residue (see Chapter I, section F.4) would be desirable. Yeast phosphoglycerate kinase contains a single, non-essential cysteinyl residue thus a reagent directed against this residue should be appropriate. With non-covalent modification of protein thiols, the reagent can migrate among reactive groups, and with covalent modification of a protein which contains many thiol residues, formation of internal disulfides can occur (Torchinskii, 1974). Since each yeast phosphoglycerate kinase molecule contains a single thiol group, migration of non-covalent reagents and formation of internal disulfides will not occur. If only one thiol group per enzyme molecule will react this requires that the probe(s) selected yield a chromogen with a high molar absorptivity. Also, a sensitive reagent will allow for more accurate determinations of low dissociation constants since it will permit the use of low enzyme concentrations. The enzyme itself does not absorb in the visible spectrum thus a chromogen absorbing in this range would be suitable. Since the results of these binding studies will be compared to kinetic studies by

others which were completed near pH 7.8 it would also be desirable for the probe to be responsive, near this pH, to changes in the environment of the thiol.

2. The Sulfhydryl Reagents

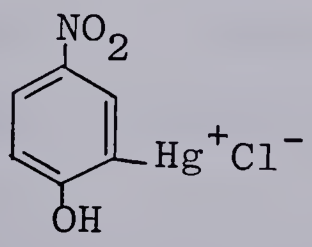
a) 2-Chloromercuri-4-nitrophenol

When metal ions react with a thiol group, mercaptide formation results as shown below (Torchinskii, 1974).

$$\text{R-SH} + \text{Me}^{+} \rightleftharpoons \text{R-SMe} + \text{H}^{+}$$

The thiol is less reactive in its protonated form (Blundell and Jenkins, 1977) and, although the reaction is reversible, the equilibrium strongly favors mercaptide formation. Reagents which react with thiols through mercaptide formation are highly specific (Torchinskii, 1974).

The unifunctional organomercurials (R-HgX eg. *p*-chloromercuribenzoate) were designed to prevent simultaneous reaction of Hg^{2+} with two nearby thiol groups and, owing to the color of the reaction product, they had the additional advantage of permitting quantitation of thiol groups (Torchinskii, 1974). In 1970 Gutfreund and McMurray described two new organomercurials. Although these workers were initially interested in such reagents for isomorphic heavy atom replacement, the potential use of their chromophoric properties as probes of protein dynamics was recognized. The structure of one of these reagents, 2-chloromercuri-4-nitrophenol, is shown below. The phenolic hydroxyl of the



free mercurial has a pK_a of 6.5 and the ionized form absorbs maximally at 405 nm (E^{mM}_{1cm} 17.4, McMurray and and Trentham, 1969). When the mercurial combines with a cysteinyl residue, its absorptivity and the degree of ionization of the phenolic hydroxyl change; these changes reflect the effect of mercaptide formation plus the effects of the new environment (local charge, hydrophobicity) (McMurray and Trentham, 1969). Since the bound chromophore retains its sensitivity to solvent, further changes in its environment will also be reflected by a change in pK_a and/ or the absorptivity of the protonated or ionized form. Thus the mercurial acts as a "reporter" group of the thiol's environment within the protein but the bound mercurial is also sensitive to further changes in this environment such as might be induced when ligands bind to the protein.

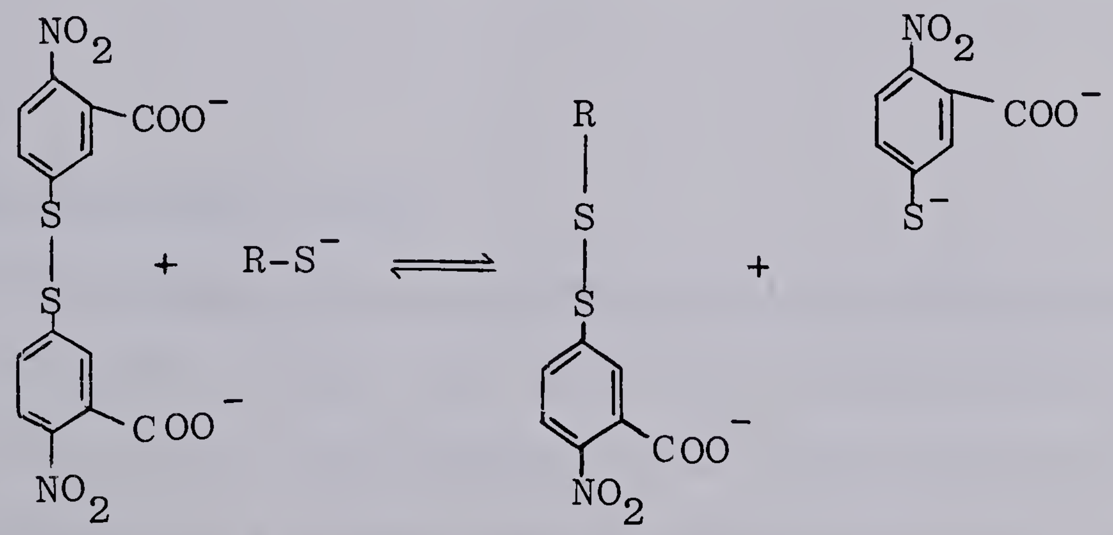
Stinson (1974) has reported a one to one stoichiometry for this reagent with yeast phosphoglycerate kinase and described the spectral properties of the protonated and ionized forms of the free and enzyme-bound mercurial. The pK_a of the phenolic hydroxyl increased from 6.60 to 8.30 following binding of the mercurial to the enzyme, and a red shift and hypochromicity of the protonated and ionized forms of the bound mercurial were noted. The pK_a increase is compatible with introduction of the mercurial into a negatively charged or relatively hydrophobic environment but, according to McMurray and Trentham (1969), increased hydrophobicity should result in hyperchromicity, at least for the free, ionized form. The specific activity and K_m

values for Mg-ATP and 3-phosphoglycerate were the same for native and mercurial-modified phosphoglycerate kinase (Stinson, 1974).

b) 5,5'-Dithiobis (2-nitrobenzoic acid)

A system to monitor ligand binding to yeast phosphoglycerate kinase which did not involve mercaptide formation was also desirable. Kinetic studies (Arvidsson and Larsson-Raźnikiewicz, 1973) indicated that thiol modification of the yeast enzyme with *p*-hydroxymercuribenzoate caused a six-fold increase in the uncompetitive K_i for Zn^{2+} with respect to Zn-ATP.

Thiol-disulfide exchange reactions are highly specific for cysteinyl residues, and the chemistry of the reaction as it would occur between DTNB and a single thiol group is shown below (Torchinskii, 1974). The rate at which the nitrothiophenolate anion is released is an indirect measure of the rate of mixed disulfide formation. The rate of reaction reflects the properties of DTNB, and the nucleophilicity and concentration of $R\text{-}S^-$.



From a technical standpoint DTNB is suitable as a reagent to monitor the effect of ligands on the rate of

phosphoglycerate kinase modification; the rate of reaction with this enzyme is relatively slow (Stinson, 1974) and can be monitored spectrophotometrically without the use of stopped-flow techniques.

B. MATERIALS AND METHODS

Materials used are listed in Chapter II, section A.

General methods used are outlined in Chapter II, section B. The rate constants for the reaction between phosphoglycerate kinase and DTNB were calculated according to the difference method as described by Gutfreund (1965).

The buffer was 5.0 mM Tes, pH 8.0 except for the trypsin and proteinase inactivation studies where mercurial-labelling and incubation mixtures were buffered with 50 mM triethanolamine, pH 7.5

C. RESULTS

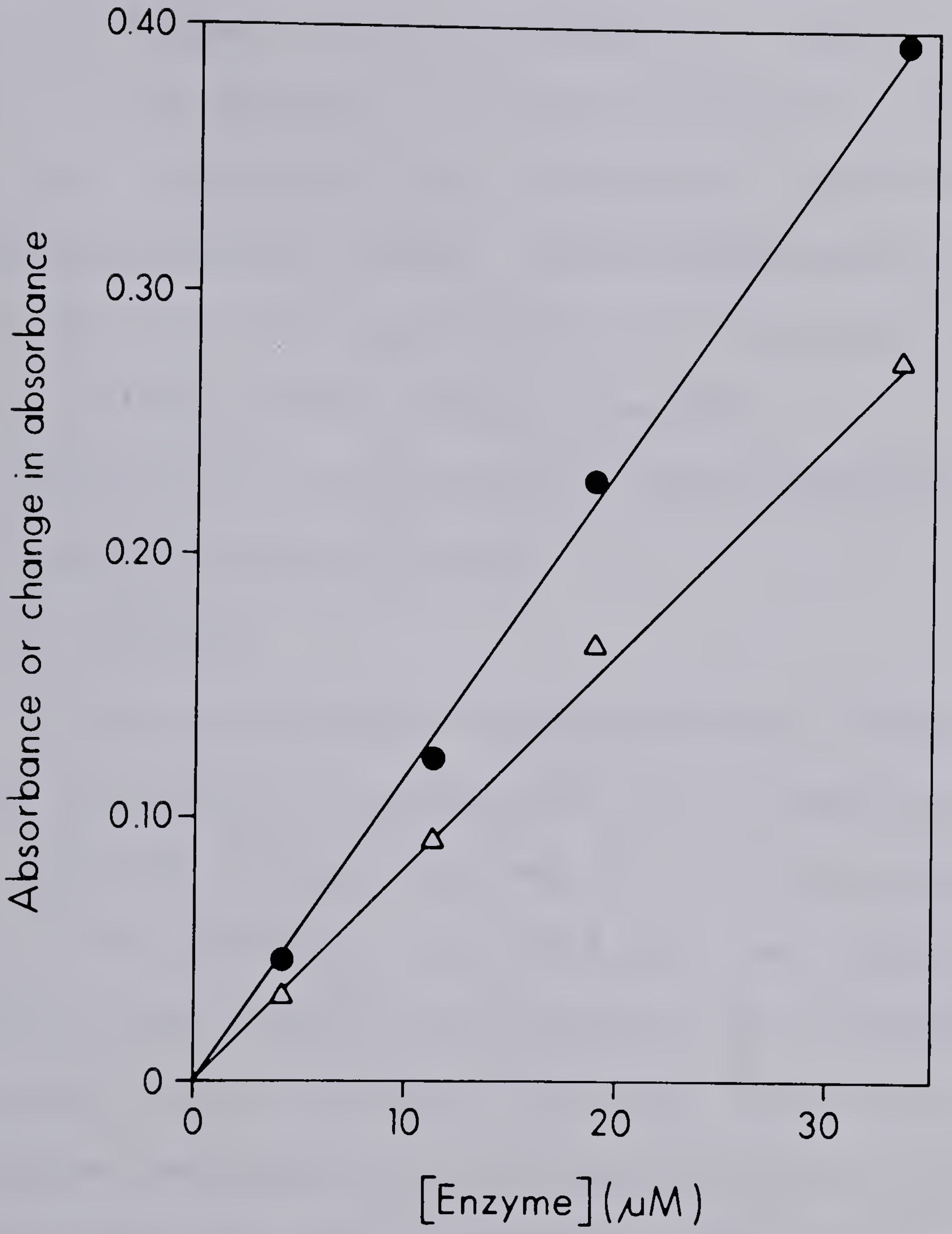
1. Dose-Response Curves

a) Phosphoglycerate kinase labelled with mercurial

Figure 1 shows that over the concentration range of 4.2 to 33.6 μM, the absorbance of the enzyme-mercurial complex increases in a linear manner. The absorbance values were corrected for the absorbance of the free, ionized form of the mercurial which was present in approximately a 5% molar excess over the enzyme concentration. This figure also shows the linear relationship between the concentration of the enzyme-mercurial complex and the maximum change in absorbance following saturation of the

FIGURE 1

The relationship between the concentration of phosphoglycerate kinase labelled with mercurial and the initial absorbance or change in absorbance following saturation with ADP. The initial absorbance values (●) were corrected for the absorbance of the excess ionized mercurial as described in the text. The decrease in absorbance following saturation with 65.7 μM ADP is also indicated (▵). The labelling and titration procedure are described in Chapter II, section B.4. The buffer was 5.0 mM Tes, pH 8.0.


0.40
0.30
0.20
0.10
0
Absorbance or change in absorbance
0
10
20
30
[Enzyme] (μM)

complex with ADP (see Chapter IV, section C.1). This corresponded to an averaged decrease of 0.0082 absorbance units per μM of labelled enzyme.

b) Modification of phosphoglycerate kinase with DTNB

The effect of DTNB concentration on the pseudo-first-order rate constants for modification of 5 μM phosphoglycerate kinase is shown in figure 2. Although the increase in rate constant is linear between 0.1 and 0.2 mM DTNB, the increase in rate constant at higher DTNB concentrations was non-linear. Rate constant plots were linear to at least 70% completion of the reaction over the range of DTNB concentrations examined.

2. Proteolytic Inactivation of Native and Modified Phosphoglycerate Kinase

a) Trypsin

Modification of phosphoglycerate kinase with mercurial increases its susceptibility to inactivation by trypsin compared to that observed with the native kinase (Fig. 3). The mercurial-labelled kinase was almost 80% inactivated after 120 min of incubation at a kinase-to-trypsin molar ratio of 500 to 1 but the activity of the native kinase decreased by less than 10% after 120 min of incubation under the same conditions. At the kinase-to-trypsin molar ratio of 500 to 1 the rate of inactivation of DTNB-modified phosphoglycerate kinase was similar to that observed with the mercurial-labelled kinase incubated under the same conditions (Fig. 3). In contrast, a kinase-

FIGURE 2

The effect of DTNB concentration on the rate of reaction between phosphoglycerate kinase and DTNB. The rate constant, k, for the reaction between 5.0 μM enzyme and DTNB was calculated as described in this chapter (section B). The experimental procedure is outlined in Chapter II, section B.5. The buffer was 5.0 mM Tes, pH 8.0.

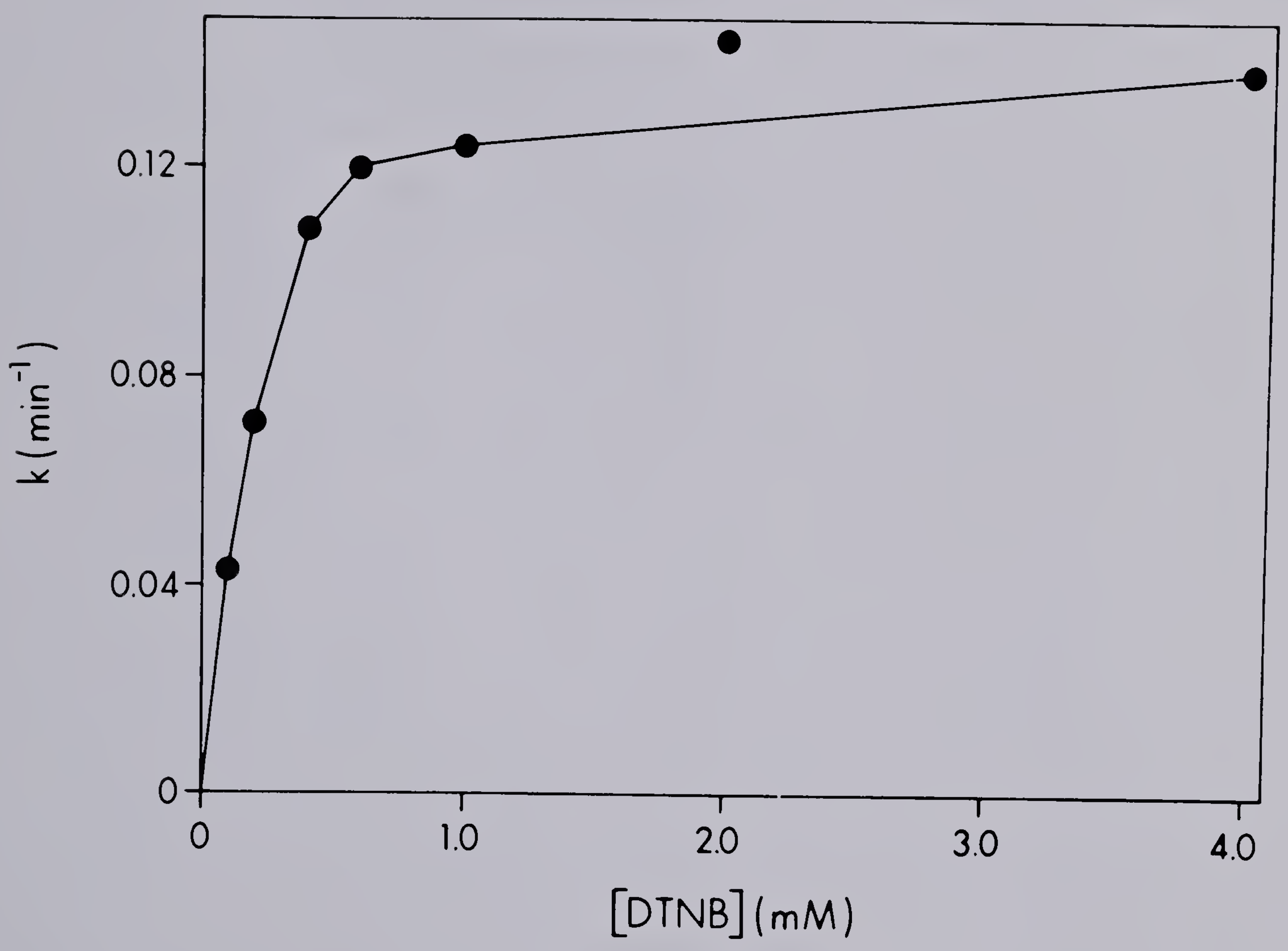
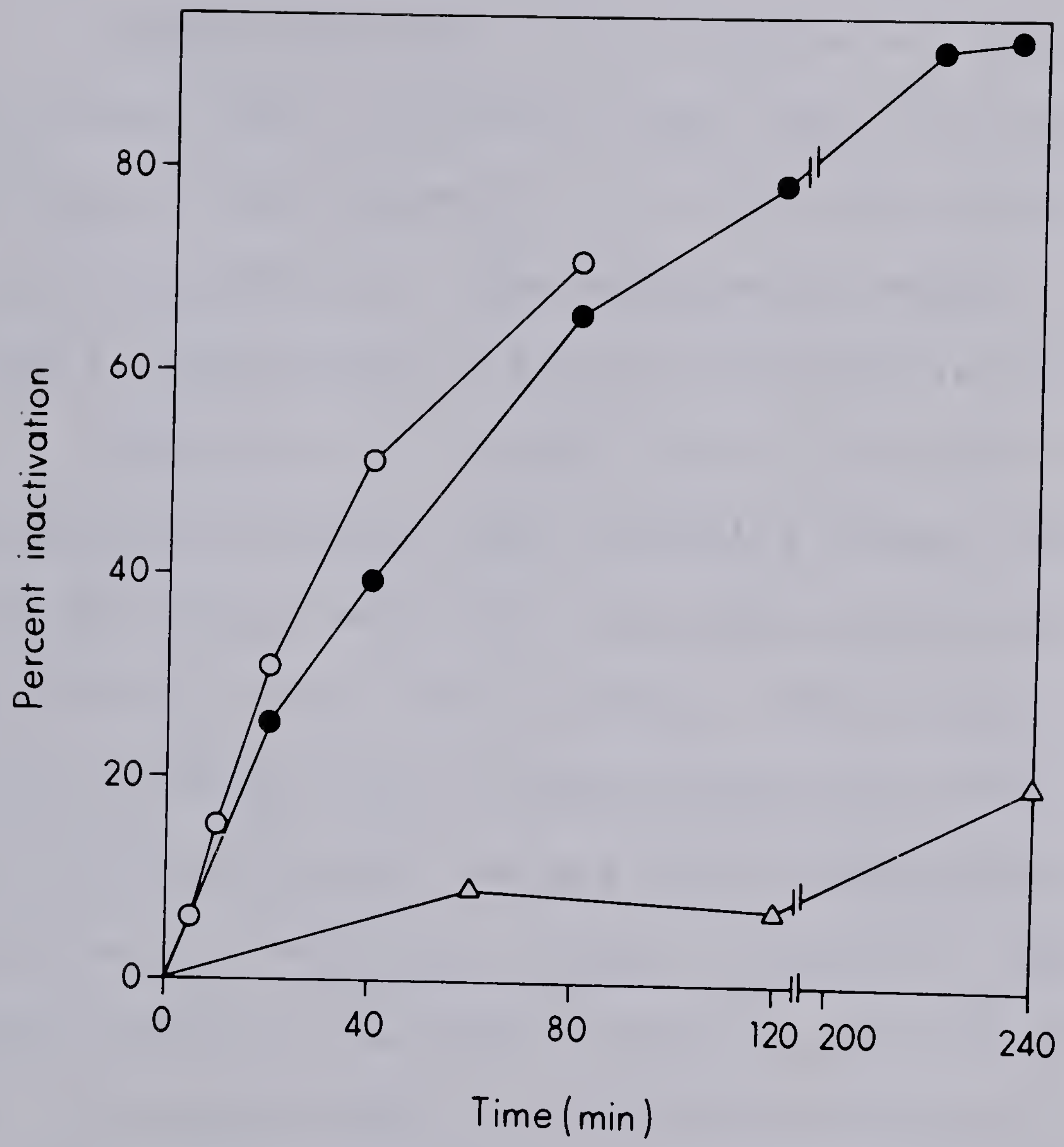

k (min^{-1})
0.12
0.08
0.04
0
0
1.0
2.0
3.0
4.0
[DTNB] (mM)

FIGURE 3

Rate of inactivation of native and mercurial-or DTNB-modified phosphoglycerate kinase by trypsin. The incubation mixture of the native (△), mercurial-labelled (●), or DTNB-modified kinase (○) was prepared as described in Chapter II, section B.7 for the modified enzyme. The mercurial-labelling technique is described in Chapter II, section B.4 and the DTNB-modification procedure is outlined in Chapter II, section B.5. The phosphoglycerate kinase-to-trypsin molar ratio was 500 to 1. Aliquots were taken at the times indicated following the addition of trypsin and assayed as described in Chapter II, section B.1. Activity is expressed as percent inactivation relative to the activity determined prior to the addition of trypsin. The buffer was 50 mM triethanolamine, pH 7.5.


Percent inactivation
0
20
40
60
80
0
40
80
120
200
240
Time (min)

to-trypsin molar ratio of 0.7 to 1 was required to obtain inactivation of the native kinase over a similar time span (data not shown). Inactivation curves of native or mercurial-labelled phosphoglycerate kinase by trypsin were similar irregardless of the presence or absence of the chymotrypsin inhibitor diphenyl carbamyl chloride in the trypsin preparation.

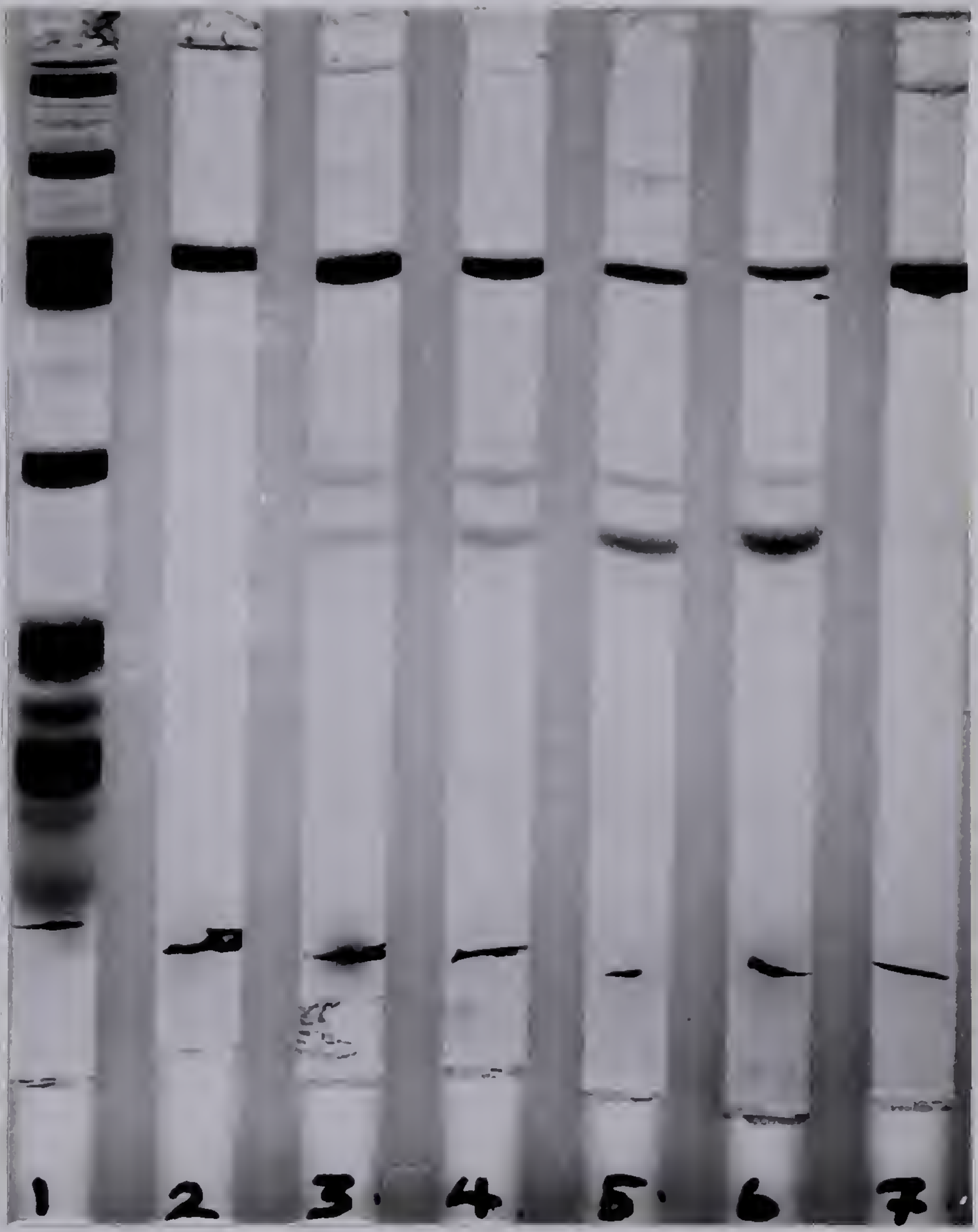
During incubation of the native and mercurial-labelled kinase (Fig. 3) samples were taken for polyacrylamide-gel electrophoresis. A molecular weight of 49,000 was determined for phosphoglycerate kinase (Fig. 4, gels 1 and 2) and agrees with the literature values (see Chapter I, section D.1). Figure 4 shows the mobilities of the polypeptides which were generated during incubation of native (gel 7) or mercurial-labelled (gels 2 to 6) phosphoglycerate kinase with trypsin. During the incubation period the concentration of phosphoglycerate kinase decreases as indicated by the gradual disappearance of the polypeptide with a molecular weight of 49,000. This disappearance appears to correlate with the rate of inactivation (Fig. 3). A polypeptide with a molecular weight of 30,000 is observed but this is a minor component which remains at a constant concentration throughout the incubation. A polypeptide with a molecular weight of 28,000 accumulates and is the major endproduct following the 2 h incubation.

b) Proteinase A

The susceptibility of mercurial-labelled phosphoglycerate kinase to inactivation by proteinase A

FIGURE 4

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate during limited tryptic digestion of native and mercurial-labelled phosphoglycerate kinase. The native and labelled kinases were incubated with trypsin as indicated in figure 3. An aliquot of the native kinase was taken at 120 min (gel 7), and aliquots of the mercurial-labelled kinase were taken at 0,20,40, 80, and 120 min (gels 2,3,4,5, and 6, respectively). The aliquots were added to sodium dodecyl sulfate as described in Chapter II, section B.9. Gel 1 is native phosphoglycerate kinase combined with the subunit molecular weight markers. The major protein bands are (top to bottom): phosphorylase *b* , 94,000; bovine serum albumin, 68,000; yeast phosphoglycerate kinase, 49,000; ovalbumin, 43,000; carbonic anhrydrase, 30,000; soybean trypsin inhibitor, 21,000; and lysozyme, 14,300. Electrophoresis and protein staining were carried out as described in Chapter II, section B.9.


1
2
3
4
5
6
7

is increased compared to that observed with the native kinase (Fig. 5). Following 120 min of incubation at a kinase-to-proteinase A molar ratio of 9.1 to 1, the labelled-kinase was 95% inactivated while the activity of the native kinase decreased by less than 5%.

During these incubations, samples were taken for polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate (Fig. 6). The decrease in concentration of the labelled kinase (gels 3 to 7) during 90 min of incubation was indicated as a decrease in the polypeptide with a molecular weight of 49,000. This decrease appeared to correlate with the loss in activity of phosphoglycerate kinase determined at the same times (Fig. 5). A polypeptide with a molecular weight of 44,000 was detected as a minor component and remained at a constant concentration throughout the incubation of the labelled kinase with proteinase A. A polypeptide with a molecular weight of 39,000 was also a minor component but its concentration decreased during the incubation. The major endproduct of proteolysis was a polypeptide with a molecular weight of 36,000 and this component accumulated during the incubation. With extended incubation of the labelled kinase a polypeptide with a molecular weight of 28,000 was observed and low molecular weight polypeptides (less than 10,000) were also apparent (gels 6 and 7).

Proteolysis of the native kinase by proteinase A to 5% inactivation (Fig. 6, gel 2) resulted in polypeptides

FIGURE 5

Rate of inactivation of native and mercurial-labelled phosphoglycerate kinase by proteinase A. The incubation mixture of native (△) or mercurial-labelled kinase (●) was prepared as described in Chapter II, section B.7 and the phosphoglycerate kinase-to-proteinase A molar ratio was 9.1 to 1. The mercurial-labelling procedure is described in Chapter II, section B.4. Aliquots were taken at the times indicated following the addition of proteinase A and assayed as described in Chapter II, section B.1. Activity is expressed as percent inactivation relative to the activity determined prior to the addition of proteinase A. The buffer was 50 mM triethanolamine, pH 7.5.

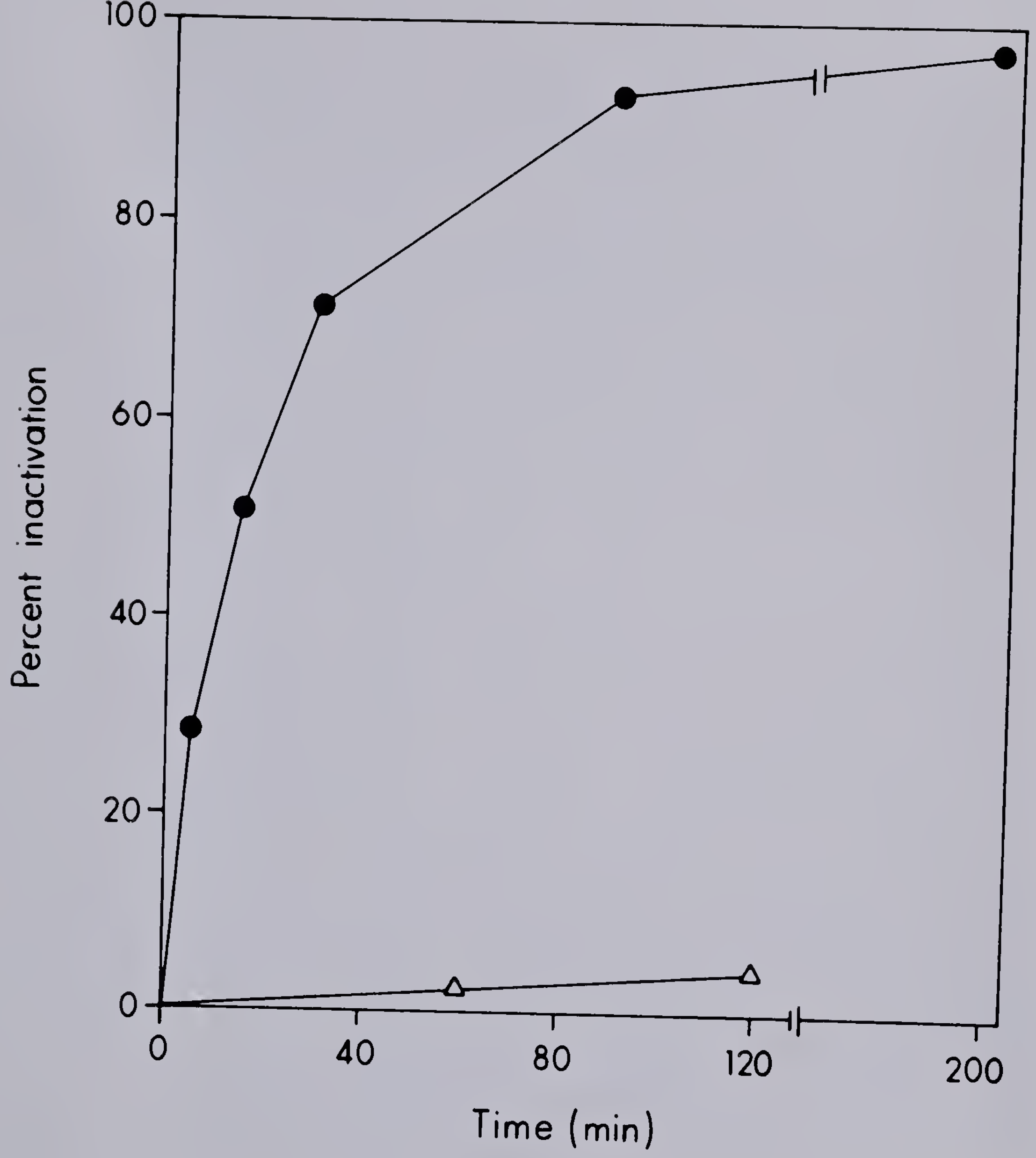
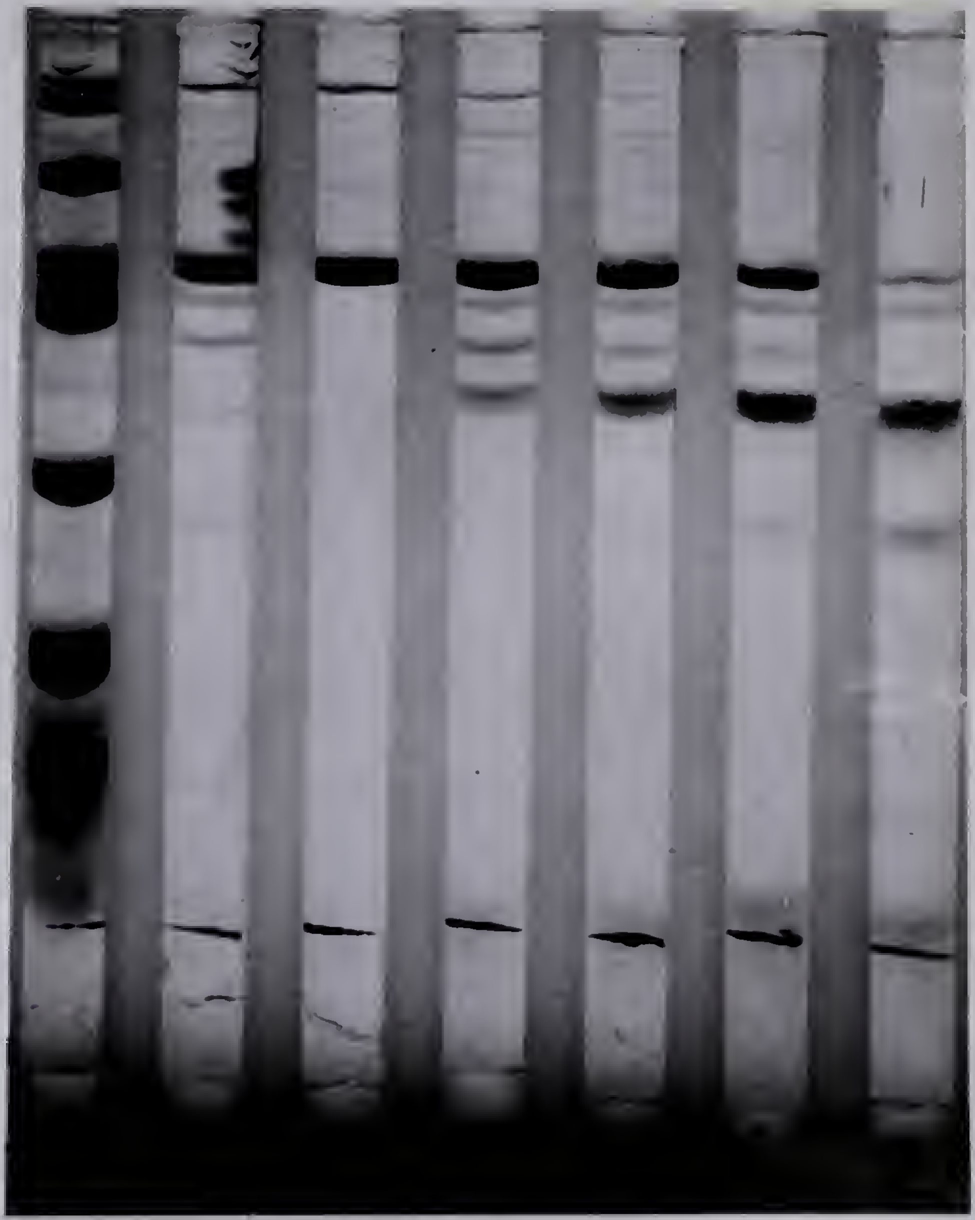

100
80
60
40
20
0
Percent inactivation
0
40
80
120
200
Time (min)

FIGURE 6

Polyacrylamide-gel electrophoresis in the presense of sodium dodecyl sulfate during limited digestion by proteinase A of native and mercurial-labelled phosphoglycerate kinase. The kinase was incubated with proteinase A as in figure 5. An aliquot of the native kinase was taken at 120 min (gel 2), and aliquots of the mercurial-labelled kinase were taken at 0,5,15,30 and 90 min (gels 3,4,5,6, and 7, respectively). The aliquots were added to sodium dodecyl sulfate as described in Chapter II, section B.9. Gel 1 shows native phosphoglycerate kinase combined with the subunit molecular weight markers which are (top to bottom): phosphorylase *b*, bovine serum albumin, (yeast phosphoglycerate kinase), ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (see legend to Fig. 4). Electrophoresis and protein staining were carried out as described in Chapter II, section B.9.



similar to those detected during incubation of the labelled kinase with proteinase A (Fig. 6, gels 4 to 7).

D. DISCUSSION

1. Phosphoglycerate Kinase Labelled with Mercurial

The absorbance of the enzyme-mercurial complex increased in a linear fashion over the concentration range of the complex used for routine binding studies (Fig. 1). These values are, however, greater than those calculated using a pK_a of 8.3 and E^{mM}_{1cm} of 15.3 at 410 nm for the mercurial bound to phosphoglycerate kinase (Stinson, 1974). This discrepency is most likely due to a change in experimental conditions; the earlier work was carried out in 5 mM triethanolamine (pH 7.5) which contained 100 mM NaCl.

Saturation of the labelled enzyme with ADP resulted in a linear relationship between the decrease in absorbance and concentration of labelled enzyme (see Chapter IV, section C.1).

2. Modification of Phosphoglycerate Kinase with DTNB

Modification of the enzyme with DTNB was completed under conditions such that pseudo-first-order kinetics should be observed. However, this was not the case for the reaction between phosphoglycerate kinase and high concentrations of DTNB (Fig. 2). The rate constant plots were linear at all DTNB concentrations examined and the calculated rate constant increased the predicted amount (~two-fold) with an increase in DTNB concentration from 0.1 and 0.2 mM. A further increase in DTNB concentration to 1.0 mM caused only

a three-fold increase in rate constant compared with the predicted increase of ten-fold. This likely indicates that DTNB, or a component in that mixture, influences the availability of the thiol for modification. The dissociation constants for certain of the anions (data not shown) and metal ions determined at 0.1 or 1.0 mM DTNB did not change (see Chapter V, section B.2).

3. Proteolytic Inactivation of Native and Modified Phosphoglycerate Kinase

Although modification of the enzyme's single thiol with mercurial does not alter K_m values (see this chapter, section 2(a)), it greatly increases the susceptibility of the kinase to inactivation by trypsin (Fig. 3). This finding confirms earlier results from this laboratory (Stinson, R.A., unpublished results) and increases to three the number of phosphoglycerate kinase preparations with which this has been observed. During limited tryptic cleavage the polypeptides generated from the native or mercurial-labelled kinase were similar (Fig. 4), and this suggests that the same mechanism(s) accounts for inactivation of the native and labelled kinase.

Modification of the kinase with mercurial increases its susceptibility to proteolytic inactivation by proteinase A (Figs. 5 and 6), and the polypeptides generated during limited proteolysis of the native or labelled kinase are the same (Fig. 6). The finding that labelling of the kinase enhances the inactivation by each endopeptidase is interesting

because of their different specificity. Trypsin hydrolyzes peptide bonds adjacent to arginyl or lysyl residues (Barman, 1969) while proteinase A shows properties similar to those of pepsin (Hata et al, 1967) in that it hydrolyzes peptide bonds adjacent to aromatic or dicarboxylic residues (Barman, 1969). The different specificity of these endopeptidases is reflected in the different molecular weights of the major polypeptides generated during limited proteolysis (Figs. 4 and 6) and suggests that contamination of proteinase A with trypsin is unlikely. The kinase-to-endopeptidase molar ratios required to inactivate mercurial-labelled phosphoglycerate kinase over a similar time span differed (Figs. 3 and 5). This could reflect the difference in specificity and/or pH optimum of trypsin (pH 7.5 to 8.0 for benzoyl arginine *p*-nitroanilide, Erlanger et al, 1961) and proteinase A (pH 3 for hemoglobin-HCl, Hata et al, 1967). Based on the amino acid composition of yeast phosphoglycerate kinase (see for example Fattoum et al, 1978) and the specificities of the two endopeptidases used here, the finding that proteolysis was limited (Figs. 4 and 6) suggests that many of the potential substrate residues of the polypeptides with molecular weights of 28,000 and 36,000 are relatively inaccessible.

According to Torchinskii (1974) the most likely result of blocking a protein's thiol group would be to prevent it from taking part in hydrophobic interactions.

The single thiol of yeast phosphoglycerate kinase could be located within a hydrophobic pocket (see this chapter, section A.2(a)). Thus it is possible that the increased susceptibility of the modified kinase to proteolytic inactivation is brought about by a conformational change which results from the inability of the thiol-containing residue to participate in hydrophobic interactions.

Modification of phosphoglycerate kinase with mercurial or DTNB caused a similar increase in susceptibility to inactivation (Figs. 3 and 5). Although the chemistries of the modification reactions differ, each introduces a substituted nitro(thio)phenolate anion adjacent to the cysteinyl residue (see this chapter, sections A.2(a) and A.2(b)). Thus modification with each reagent could cause the same disturbance of local intramolecular bonding adjacent to the cysteinyl residue and, in doing so, make the critical peptide bonds available for cleavage through conformational changes. The use of probes structurally unrelated to the mercurial and DTNB would be required to determine the relative importance of blocking of the sulfhydryl or the nature of the incoming reagents as the cause of the increased susceptibility reported here.

According to Szabolsci et al (1959) blocking of the thiol groups of muscle glyceraldehyde 3-phosphate dehydrogenase induced changes in the secondary and tertiary structure of the enzyme. These changes resulted

in an increased susceptibility of the modified enzyme to tryptic hydrolysis. Szabolsci and Bisku (1961) have reported similar results with muscle aldolase. However, according to the same reports, catalytic activity of the fully modified form of each enzyme was abolished. In contrast, modification of the single thiol of yeast phosphoglycerate kinase does not change its catalytic activity.

CHAPTER IV

ANION BINDING TO PHOSPHOGLYCERATE KINASE

A. INTRODUCTION

Previous studies (Stinson, 1974) showed that 3-phosphoglycerate and inorganic phosphate decreased the rate constant for the reaction between the enzyme's single thiol and the organomercurial 2-chloromercuri-4-nitrophenol. Meyer and Westhead (1976) reported that sulfate slowed the rate of thiol modification by tetranitromethane. Both of these studies indicated that the substrates or substrate analogs could influence thiol availability. Although X-ray crystallographic studies indicated that this residue was 30 Å removed from, and on the opposite lobe to the active center (see Chapter I, section D.3), it was conceivable that substrate binding to the active center in solution could cause conformational changes in the enzyme such that the environment of the thiol was altered. That this in fact did occur was shown by an increase in pK_a of the mercurial's phenolic hydroxyl determined in the presence of substrates (Stinson, 1974). The present study was initiated to quantitate substrate binding to the active center of the mercurial-labelled enzyme. However, since kinetic work suggested that a variety of anions activated and inhibited catalytic activity of the yeast enzyme (Larsson-Raźnikiewicz and Jansson, 1973; Scopes, 1976), the studies were extended to include non-substrate anions.

As a second system to monitor the binding of substrate and non-substrate anions, we chose the reaction between native phosphoglycerate kinase and DTNB. A method to determine the dissociation constant for each anion is presented.

The susceptibility of phosphoglycerate kinase to proteolytic inactivation by bovine pancreatic trypsin or yeast proteinase A is increased when the kinase is modified with the mercurial or DTNB (see Chapter III, sections C.2(a) and C.2(b)). The effect of the divalent anion, succinate, on the susceptibility of the native and mercurial-labelled kinase to proteolytic inactivation by trypsin and proteinase A, respectively was also examined.

B. MATERIALS AND METHODS

1. Materials

General materials used are listed in Chapter II, section A. The following were obtained from Sigma Chemical Co. (St. Louis, Mo.): the monosodium salt of D(-)3-phosphoglycerate (free of 2,3-bisphosphoglycerate), NADH, the bisTris salt of 2,3-bisphosphoglycerate, and the sodium salts of citrate, ATP, ADP, AMP, oxalate, succinate and 2-phosphoglycerate. Sulfate (disodium salt), phosphate (monosodium salt), acetate (monosodium salt), $MgCl_2$, and thioglycollic acid were obtained from Fisher Scientific (Fairlawn, N.J.). Analytical reagent grade pyrophosphate was obtained as the tetrasodium salt from B.D.H. (Poole, England), and succinamide was from Eastman Kodak Co. (Rochester, N.Y.).

For the spectrophotometric binding studies the buffer was 5.0 mM Tes, pH 8.0. Stock solutions of anions were prepared in buffer and brought to a final pH of 8.0 with sodium hydroxide or hydrochloric acid. Proteolytic inactivation studies were buffered in 50 mM triethanolamine adjusted to pH 7.5 with sodium hydroxide. Stock solutions of succinate were prepared in this buffer and brought to a final pH of 7.5 with sodium hydroxide and hydrochloric acid.

2. Methods

Ionization constants for the following acids were obtained from the CRC Handbook of Biochemistry (Sober, 1968): citric, oxalic, succinic, phosphoric, glycerol 2-phosphoric, acetic, and sulfuric; those for ATP, ADP, and AMP were from Perrin (1965). Ionization constants for 3-phosphoglyceric acid, 2,3-bisphosphoglyceric acid, and pyrophosphoric acid were as reported by Larsson-Raźnikiewicz (1972), Yap and Saroff (1971) and Moe and Wiest, 1977), respectively. The anion charge at pH 8.0 was calculated by use of the Henderson-Hasselbach equation.

a) Titrations of mercurial-labelled phosphoglycerate kinase with anions

General methods used are outlined in Chapter II, section B.

Titrations of the enzyme-mercurial complex with certain of the divalent anions and all of the monovalent anions were done in the presence of 1.0 mM $MgCl_2$ (see this chapter, section C.1). The binding affinity of certain anions

that chelate Mg^{2+} was determined in the presence of 10 mM $MgCl_2$. The dissociation constants for the Mg-complexes of 2,3-bisphosphoglycerate, ATP, and pyrophosphate were 0.13 mM (Achilles et al, 1972), 0.08 mM (Larsson-Raźnikiewicz, 1967), and 0.9 mM (Lambert and Watters, 1957), respectively.

For the tighter binding anions, a Scatchard plot was used to determine the dissociation constant and the number of anion binding sites per molecule of the enzyme labelled with mercurial. Dissociation constants of 1.0 mM or greater were obtained from double-reciprocal plots.

b) Rate of reaction between phosphoglycerate kinase and DTNB in the presence of anions

The dissociation constants for phosphoglycerate kinase and certain anions were also determined by the effect of each anion on the rate constant for the reaction between the enzyme and DTNB. The pseudo-first-order rate constants were calculated by the endpoint method as described by Gutfreund (1965) and converted to second-order rate constants. The dissociation constant was obtained from a plot of the reciprocal of the second-order rate constant (ordinate) *vs.* anion concentration, and was the negative of the intercept on the abscissa. See Chapter II, section B for general methods.

c) Inactivation of phosphoglycerate kinase by trypsin or proteinase A in the presence of anions

General methods used are outlined in Chapter II, section B. The kinase was labelled in the presence of

50 mM triethanolamine, pH 7.5.

The effect of succinate on the susceptibility of native phosphoglycerate kinase to inactivation by trypsin was carried out as described in Chapter II, section B.7 for the native kinase. The effect of succinate on the inactivation of the mercurial-labelled kinase by proteinase A was according to the protocol outlined for the mercurial-labelled kinase (see Chapter II, section B.7).

C. RESULTS

1. Titrations of Mercurial-Labelled Phosphoglycerate Kinase with Anions

The decrease in absorbance resulting from titration of the enzyme-mercurial complex with each anion investigated yielded an apparent binding curve; those obtained for citrate and ADP are shown in figure 7A. The decrease in absorbance could result from an increase in pK_a of the mercurial's phenolic hydroxyl or it could be due to a decrease in the molar absorptivity at 410 nm of the ionized form of the bound mercurial (see Chapter III, section A.2(a)). The Scatchard plots of the anion binding data were linear (Fig. 7B) and extrapolated to one anion binding site per enzyme molecule. The dissociation constants and the number of anion binding sites determined for ADP were unchanged over the concentration range of 4.2 to 33.6 μM of the enzyme-mercurial complex.

The binding affinity of several anions was verified with two commercial sources and one laboratory preparation of yeast phosphoglycerate kinase. Addition of anions to a

FIGURE 7

Citrate and ADP titrations of phosphoglycerate kinase labelled with mercurial. (A) Binding curves; (B) Scatchard plots of the data from (A). The ADP (●) and citrate (▲) titrations were of 22.5 μM and 20.1 μM of the enzyme-mercurial complex, respectively, and were carried out as described in Chapter II, section B.4. The fraction of the maximum change in absorbance at 410 nm obtained with each addition of anion is r, and the concentration of free anion is u. The buffer was 5.0 mM Tes, pH 8.0.

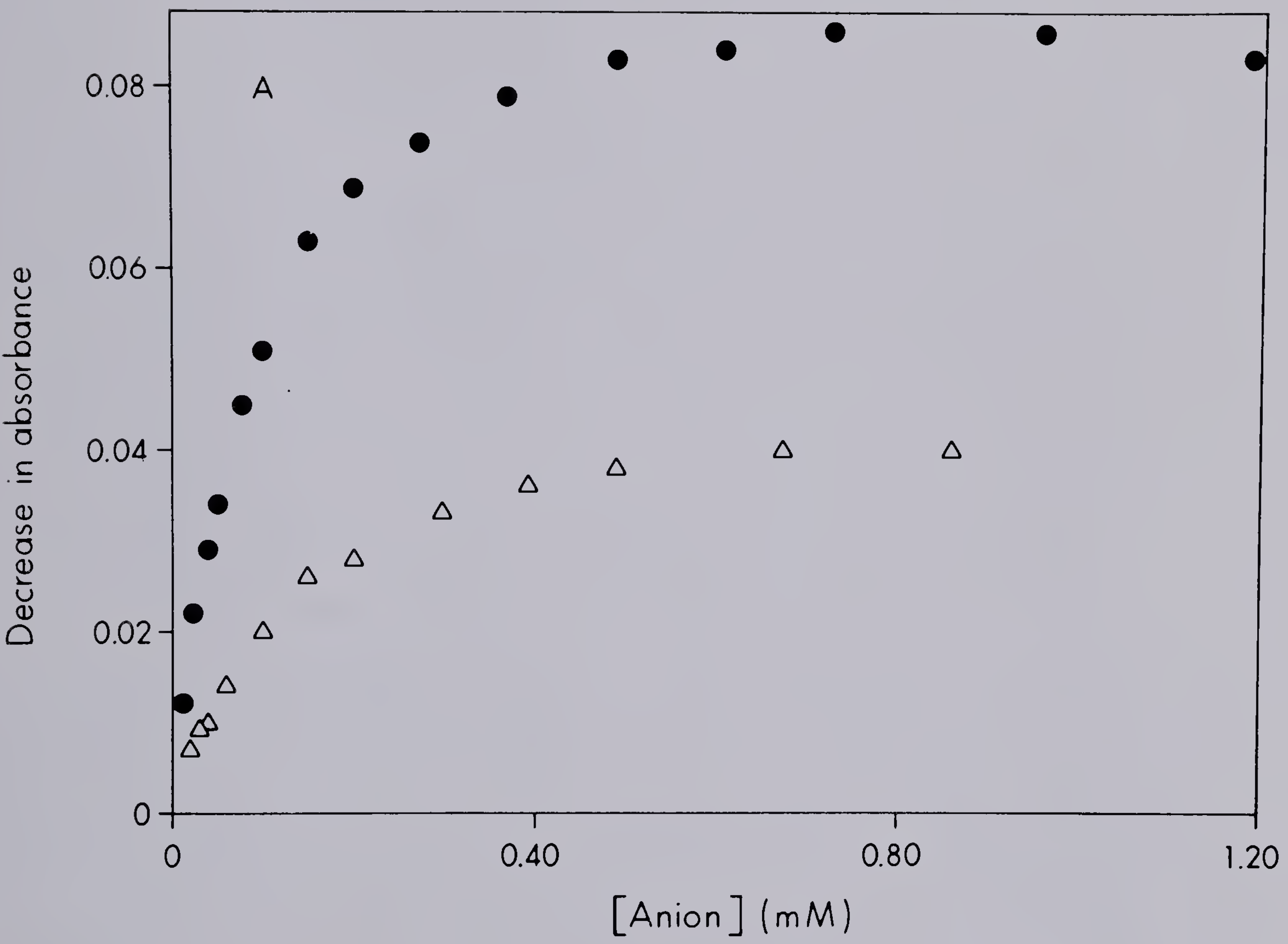
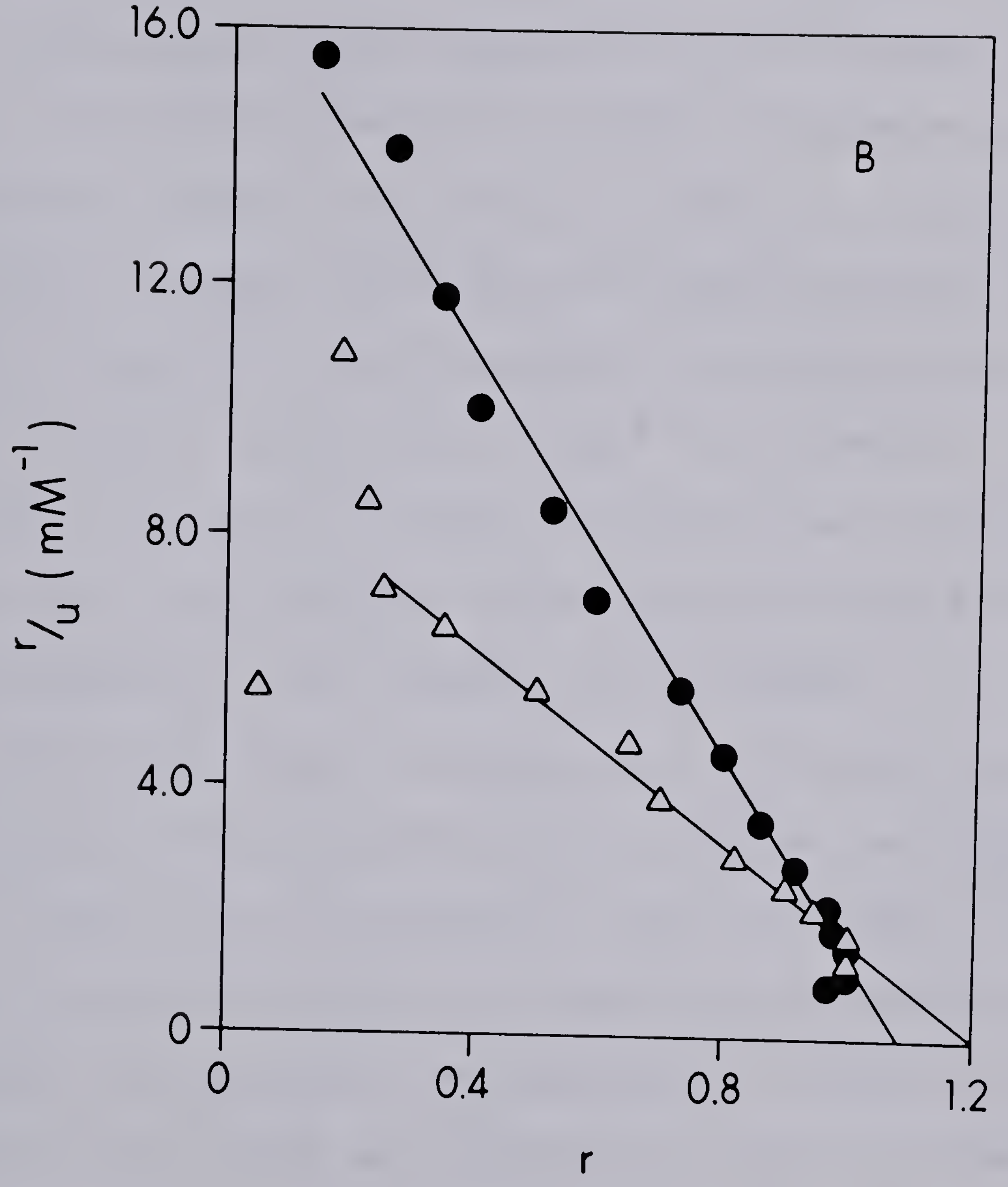

A
Decrease in absorbance
0
0.02
0.04
0.06
0.08
0
0.40
0.80
1.20
[Anion] (mM)

FIGURE 7

Citrate and ADP titrations of phosphoglycerate kinase labelled with mercurial. (A) Binding curves; (B) Scatchard plots of the data from (A). The ADP (●) and citrate (▲) titrations were of 22.5 μM and 20.1 μM of the enzyme-mercurial complex, respectively, and were carried out as described in Chapter II, section B.4. The fraction of the maximum change in absorbance at 410 nm obtained with each addition of anion is r, and the concentration of free anion is u. The buffer was 5.0 mM Tes, pH 8.0.


16.0
12.0
8.0
4.0
0
$^r/_u$ (mM^{-1})
0
0.4
0.8
1.2
r
B

complex of thioglycollic acid and 2-chloromercuri-4-nitrophenol failed to alter the absorbance of the latter at 410 nm. This suggests that the absorbance change of the mercurial bound to phosphoglycerate kinase reflects binding of the anion to a site on the enzyme. It is unlikely that this effect is caused by cations since the sodium salts of most anions were used.

The dissociation constants and the number of binding sites determined for a number of substrate and non-substrate anions are listed in Table 1. The binding affinities of anions which do not tightly chelate Mg^{2+} were not affected by the presence of the cation at a level of 1.0 mM. This level of Mg^{2+} did however, prevent an initial increase in absorbance which was consistently apparent with the divalent and monovalent anions and had been attributed to Na^+ binding to the enzyme.

The much weaker binding of the divalent and monovalent anions meant that saturation of the enzyme was impossible without significant increases in ionic strength. This could lead to considerable error in end-point determination through ionization suppression of the mercurial's phenolic hydroxyl. Scatchard plots based on estimation of the experimental end points yielded up to 1.8 anion binding sites per molecule of enzyme. However, end points calculated from the ordinate intercept of the double-reciprocal plots (see this chapter, section B.1) were consistently higher than those estimated, and resulted in Scatchard plots which extrapolated to one binding site per enzyme molecule. The calculated end

TABLE 1

Binding Parameters for Anions Determined from Titrations of Phosphoglycerate Kinase Labelled with Mercurial

The number of determinations of the dissociation constant is given in parentheses. Dissociation constants from repeat titrations with the same anion differed by less than a factor of two. Results for Mg-ATP were determined in the presence of 10.0 mM $MgCl_2$, and those for sulfate, 2-phosphoglycerate, phosphate, succinate, bromide, chloride, and acetate in the presence of 1.0 mM $MgCl_2$. Values omitted in the last two columns could not be accurately determined (see this chapter, section C.1).

Anion	Negative charge at pH 8.0	Dissociation constant	Anion bound to enzyme	Maximum chromogenicity decrease
		mM	mol/ 47,000 g	ΔA/μM
2,3-Bisphosphoglycerate	4.1	0.00042(4)	1.01	0.0054(4)
ATP	4.0	0.00190(6)	1.04	0.0046(6)
Pyrophosphate	3.2	0.0105 (5)	1.02	0.0049(5)
3-Phosphoglycerate	3.0	0.0327 (3)	1.05	0.0035(3)
ADP	3.0	0.0610 (4)	1.15	0.0030(3)
Citrate	3.0	0.138 (4)	1.19	0.0019(4)
AMP	2.0	1.35 (3)	-	-
Sulfate	2.0	1.70 (2)	-	-
Oxalate	2.0	2.63 (3)	-	-
Mg-ATP	2.0	2.70 (1)	-	-
2-Phosphoglycerate	2.0	3.28 (4)	-	-
Phosphate	2.0	4.20 (2)	-	-
Succinate	2.0	13.6 (2)	-	-
Bromide	1.0	48.0 (1)	-	-
Chloride	1.0	106 (2)	-	-
Acetate	1.0	182 (1)	-	-

point is likely the more accurate, but in any case, the method of arriving at the end point did not significantly alter the dissociation constant.

Anion binding becomes considerably tighter as the charge on the anion is increased and a linear relationship appears to exist between the negative logarithm of the dissociation constant and the net anion charge (Fig. 8). The slope of the line in figure 8 is 1.7. In other words, each unit increase in charge of an anion will increase its affinity for phosphoglycerate kinase by a factor of 50 (antilog of 1.7). It is interesting that the dissociation constant for Mg-ATP is in agreement with those obtained for other divalent anions and is more than 1000-fold weaker than that for tetravalent ATP.

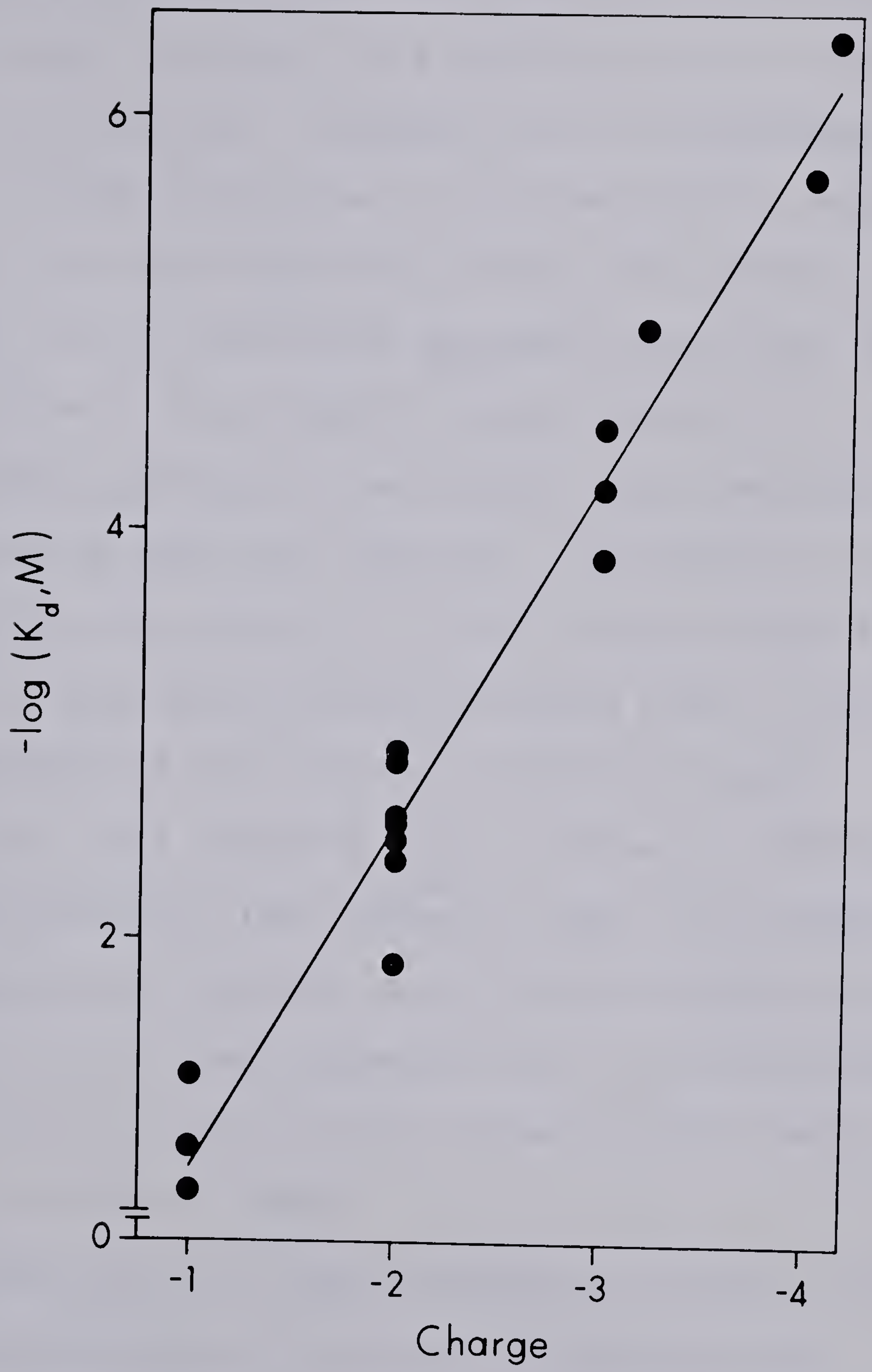
It was not possible to accurately determine the dissociation constants between Mg-2,3-bisphosphoglycerate or Mg-pyrophosphate and phosphoglycerate kinase. The cation binds to the totally ionized forms of both anions much more strongly than to the monohydrogen forms, and at pH 8.0 a considerable amount of the latter form exists for both anions. Thus one could not be certain at low concentrations of the anion that it was fully complexed with Mg^{2+}. Nevertheless, titrations in the presence of 10 mM $MgCl_2$ resulted in an averaged (both anions) increase of 700-fold in the anion-enzyme dissociation constant.

2. Rate of Reaction between Phosphoglycerate Kinase and DTNB in the Presence of Anions

Anions were found to decrease the rate of reaction

FIGURE 8

The relationship between anion charge and the negative logarithm of its dissociation constant with phosphoglycerate kinase labelled with mercurial. Each point is an average of the dissociation constants, K_d, for individual anions as listed in Table 1.


-log (K$_d$, M)
6
4
2
0
-1
-2
-3
-4
Charge

between the single thiol of native phosphoglycerate kinase and DTNB, and this was suggestive that the anions were binding to the enzyme. A plot of the reciprocal of the second-order rate constant against anion concentration resulted in a straight line that could be extrapolated to the abscissa intercept to yield the negative of the dissociation constant. Examples for 2,3-bisphosphoglycerate and ATP are shown in figure 9 and dissociation constants determined for these and other anions are listed in Table 2. The values are in reasonable agreement with those determined from titration of the labelled enzyme (Table 1). A plot of the negative logarithm of the dissociation constant *vs.* the anion charge is shown in figure 10. The slope of the line drawn through the points is 1.5 and compares well with the value of 1.7 obtained from an analogous plot of the data from titrations of the mercurial-labelled enzyme.

Using 14.1 as the E^{mM}_{1cm} at 412 nm of 3-carboxylato-4-nitrothiophenolate ion (Collier, 1973), the number of thiol residues was calculated as 0.8 per enzyme molecule. This value is in close agreement with those previously reported for the yeast enzyme (Larsson-Raźnikiewicz, 1970a; Krietsch and Bücher, 1970).

3. Inactivation of Phosphoglycerate Kinase by Trypsin or Proteinase A in the Presence of Anions

Phosphoglycerate kinase labelled with the mercurial is more susceptible to proteolytic inactivation by trypsin or proteinase A than the native kinase (see Chapter III, sections C.2(a) and C.2(b)). Succinate decreased the

FIGURE 9

The effect of 2,3-bisphosphoglycerate and ATP on the rate of reaction between phosphoglycerate kinase and DTNB. The effect of 2,3-bisphosphoglycerate (●) was evaluated at 10.0 μM phosphoglycerate kinase and 1.0 mM DTNB while the effect of ATP (△) was monitored at 5.0 μM phosphoglycerate kinase and 2.0 mM DTNB. The experimental procedure is outlined in Chapter II, section B.5. The rate constants, k, were calculated as described in this chapter, section B.2(b). The buffer was 5.0 mM Tes, pH 8.0.

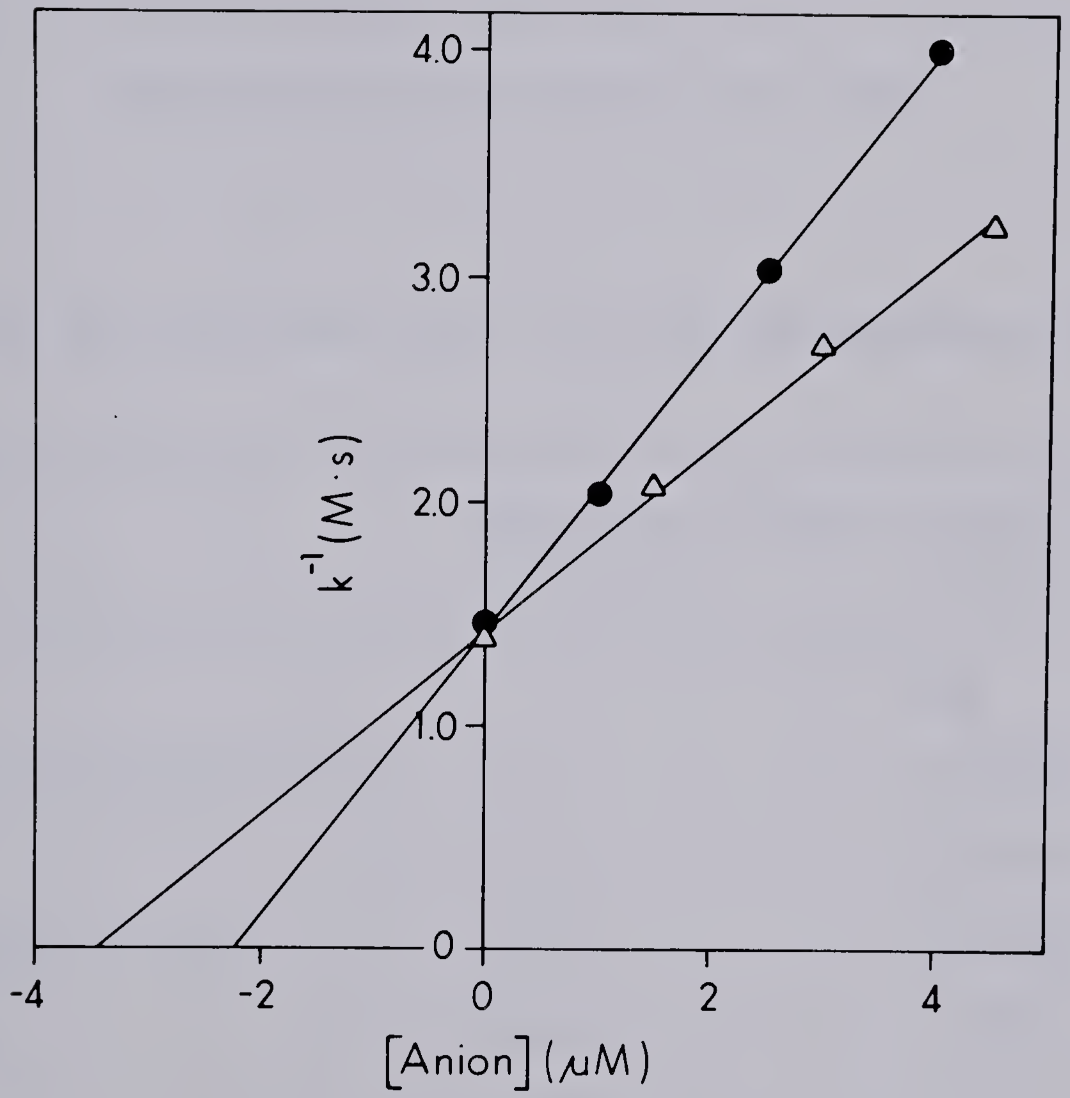

4.0
3.0
2.0
1.0
0
k^{-1} (M·s)
-4
-2
0
2
4
[Anion] (μM)

TABLE 2

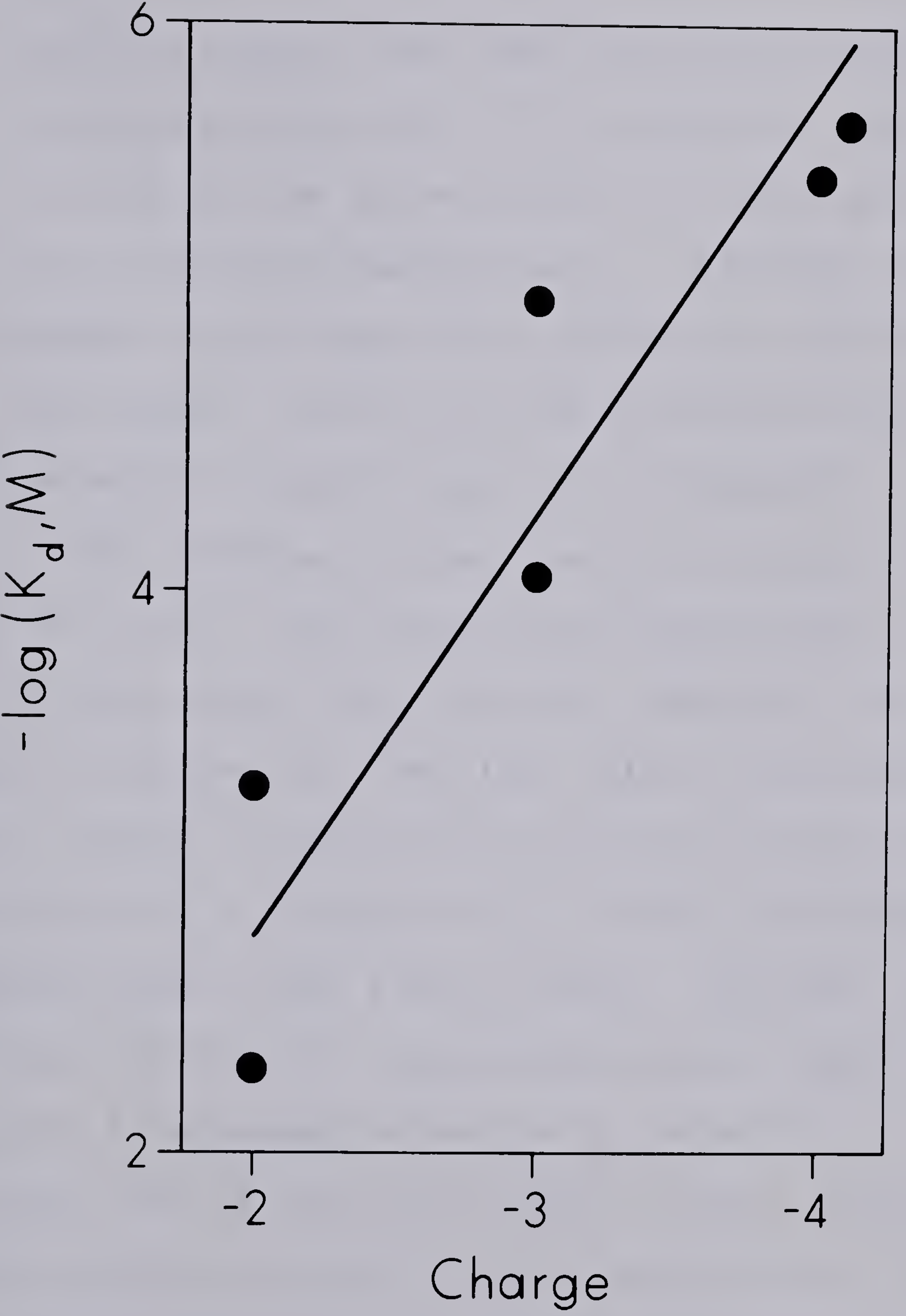
Dissociation Constants for Anions Determined by the Decrease in Rate Constant for the Reaction between Phosphoglycerate Kinase and DTNB

No change was obtained with 10 or 20 mM succinamide.

Anion	Negative charge at pH 8.0	Dissociation constant
		mM
2,3-Bisphosphoglycerate	4.1	0.0022
ATP	4.0	0.0034
3-Phosphoglycerate	3.0	0.0093
Citrate	3.0	0.085
AMP	2.0	0.5
Succinate	2.0	5.0
Succinamide	0	-

FIGURE 10

The relationship between anion charge and the negative logarithm of its dissociation constant with phosphoglycerate kinase. Dissociation constants, K_d, for the individual anions are listed in Table 2.


-log (K_d,M)
6
4
2
-2
-3
-4
Charge

susceptibility of the labelled kinase to inactivation by proteinase A and this is shown in figure 11. Succinate at 30 mM also decreased the susceptibility of native phosphoglycerate kinase to inactivation by trypsin (data not shown).

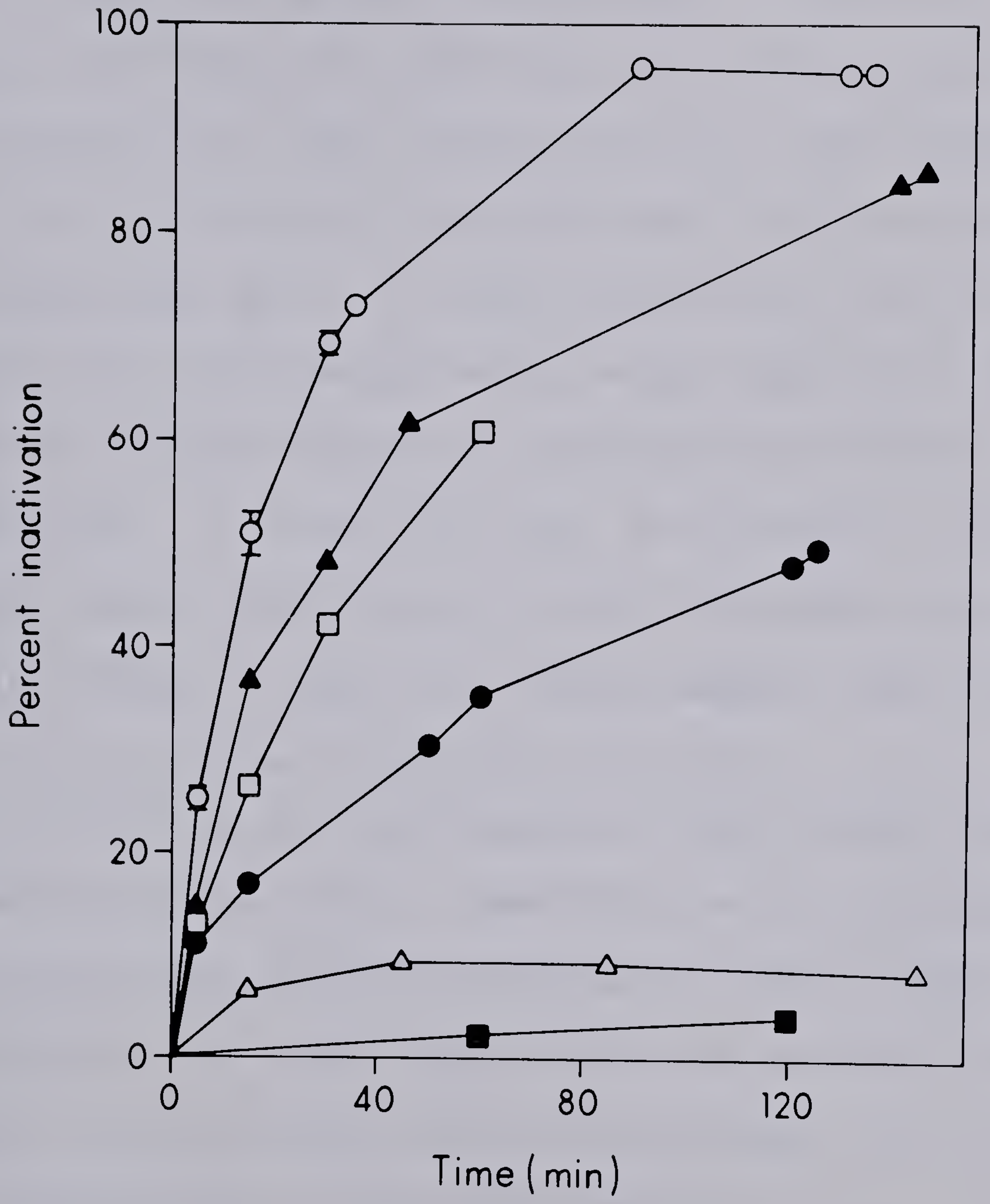
D. DISCUSSION

These results suggest that yeast phosphoglycerate kinase contains an anion binding site. It is unlikely that the binding of anions to the active center is being monitored because of the following observations. Firstly, under our experimental conditions the dissociation constant for free ATP (Tables 1 and 2) is approximately three orders of magnitude tighter than that for Mg-ATP (Table 1). This finding contrasts with the report by Chapman et al (1977) that Mg-ATP binds more tightly to the active center than does free ATP. Secondly, the dissociation constant for free ADP (Table 1) is approximately two orders of magnitude tighter than the value of 3.2 mM reported by Chapman et al (1977) for binding of this nucleotide to the active center. Thirdly, the dissociation constant for 3-phosphoglycerate (Tables 1 and 2) is not in agreement with its K_m value of approximately 0.65 mM (see for example Scopes, 1973) which should be the case for a rapid equilibrium, random mechanism as has been proposed for this enzyme (see Chapter I, section F.5).

The tightness of anion binding appears to be determined primarily by charge (Tables 1 and 2; Figs. 8 and 10). Since only anions with closely spaced charges were examined comments as to the dimensions of the site cannot be made.

FIGURE 11

The effect of succinate on the rate of inactivation of phosphoglycerate kinase by proteinase A. The succinate concentrations were 5.0 mM (▲), 10.0 mM (□), and 30.0 mM (●). The rate of inactivation of the native (■) and mercurial-labelled (○) kinase is also shown, and (△) indicates the decrease in activity of the mercurial-labelled kinase in the absence of proteinase A. The incubation mixtures were prepared as described in Chapter II, section B.7 and the phosphoglycerate kinase-to-proteinase A molar ratio was 9.1 to 1. Aliquots were taken at the times indicated following the addition of proteinase A and assayed as described in Chapter II, section B.1. Activity is expressed as percent inactivation relative to the activity determined prior to the addition of proteinase A. The buffer was 50 mM triethanolamine, pH 7.5. The bars indicate the range of duplicate runs.


100
80
60
40
20
0
Percent inactivation
0
40
80
120
Time (min)

On the basis of charge specificity it is likely that the binding site contains a cluster of positively charged residues. Thus, a pH-dependence of the anion-binding process should be observed and this may in part account for the findings of Tanswell et al (1974) who report two Mg-ATP binding sites per enzyme molecule at pH 7.8 but only one Mg-ATP binding site at pH 6.3.

Under physiological conditions the anions most likely to be occupying the anion binding site of the cytosolic enzyme in most cells are the substrates, particularly in their uncomplexed form. In the erythrocyte free and Mg-complexed 2,3-bisphosphoglycerate might also bind while the mitochondrial enzyme could be influenced by citrate and the substrates. A large trivalent anion such as ADP binds with equal affinity as a small trivalent anion such as citrate (Table 1) and this could suggest that the anion binding site is in an accessable location.

It is unlikely that the enzyme's thiol forms part of the anion binding site. Modification of the enzyme with the mercurial or DTNB introduces a substituted nitrothiophenolate anion adjacent to the cysteinyl residue. But, with the mercurial, modification occurs prior to anion binding while in the DTNB system it occurs subsequent to anion binding. The dissociation constants for the anions determined by the two methods are in close agreement (Tables 1 and 2) when one considers the two-fold error associated with repeat titrations of the mercurial-labelled enzyme. The pseudo-first-order rate constant plots for the DTNB reaction in the presence of anions remain linear. These

results indicate that the order of modification or the chemistry of the modification reactions (see Chapter III, sections A.2(a) and A.2(b)) does not change the enzyme's affinity for anions and rules out direct involvement of the cysteinyl residue in anion binding. The susceptibility of mercurial-labelled phosphoglycerate kinase to inactivation by proteinase A is decreased in the presence of succinate at concentrations compatible with its binding to the anion binding site (Fig. 11). This also suggests that anions bind to the mercurial-labelled enzyme.

Steric hinderance alone cannot account for the decreased availability of the enzyme's thiol for modification with DTNB in the presence of anions. At half-saturation of the anion binding site with a variety of individual anions, second-order rate constants are identical, and this suggests that the same degree of occupancy of the anion binding site produces the same change despite the charge of the anion. Indeed, with the DTNB system the method used here to determine dissociation constants for the anions (Fig. 9) is based on the assumption that DTNB and the anions bind non-competitively. The above findings suggest that the two thiol-directed probes monitor conformational changes of the enzyme which occur when anions bind.

The early reports of two substrate binding sites per enzyme molecule (see Chapter I, section F.2) could be explained in terms of the ability of the substrates to bind at the active site and at the anion binding site described here. The ^{31}P NMR substrate binding study of Rao and

co-workers (1978) indicates that two molecules of ATP are bound per molecule of the yeast enzyme. Binding at one site is weakened in the presence of Mg^{2+} thus this site appears to show the same property as the anion binding site reported here (Table 1).

Occupancy of the anion binding site described here could also account for the reports by others of substrate activation (see Chapter I, section F.2; Larsson-Raźnikiewicz and Shierbeck, 1977) and anion activation (Larsson-Raźnikiewicz and Jansson, 1973; Scopes, 1976; Scopes, 1978a). Since substrates and other anions can bind to the putative regulatory site it is important to know for both kinetic and binding studies the composition of the buffer, pH, and whether salts may be present.

Scopes (1978b) reports two Mg^{2+}-nucleotide binding sites per enzyme molecule and it is likely that the low affinity binding sites reflect binding of these substrates at the anion binding site. A single 3-phosphoglycerate or 1,3-bisphosphoglycerate binding site was demonstrated and dissociation constants of 32 and 0.05 μM, respectively, were reported. However, it should be noted that Scopes currently uses 3-phosphoglycerate in an affinity elution step during enzyme purification, and it is possible that it could be blocking the sugar substrate binding site at the active center despite "extensive" dialysis. The dissociation constant determined by Scopes for 3-phosphoglycerate agrees with that reported here, but his value for 1,3-bisphosphoglycerate is one order of magnitude lower than the dissociation constants for other tetravalent

anions reported here (Tables 1 and 2). This latter discrepancy probably reflects the increased sensitivity of Scopes' method. Although the author argues that the ability of 3-phosphoglycerate to weaken 1,3-bisphosphoglycerate binding indicates that binding is to the active center, the same effect could also occur with competition at the anion binding site. The three-fold increase in the dissociation constant for 3-phosphoglycerate in the presence of 5 mM sulfate reported by Scopes could also occur with competition between this substrate and sulfate at the anion binding site.

Because of the ability of anions to activate and inhibit phosphoglycerate kinase, Scopes (1978b) also examined the effect of sulfate and phosphate on substrate binding. Sulfate weakened the binding of Mg-ATP and Mg-ADP to their high affinity (active center) binding sites, and weakened the binding of the sugar substrates. This effect was competitive with respect to Mg-ATP but not strictly competitive with respect to the other three substrates, and two sulfate binding sites with dissociation constants of 1.6 mM, and 7 to 150 mM were indicated. Scopes reports a marked effect of ionic strength on sulfate binding; the dissociation constant of 1.6 mM at I=0.15 becomes 50 μM at I=0.015. Similar results were obtained with phosphate. Based on the results of his substrate and anion binding studies, Scopes has proposed an activatory mechanism which places the anion binding activatory site at the active center.

X-ray and NMR studies indicate that sulfate binds to the

active center (see Chapter I, section D.3) with saturation occurring at less than 0.5 mM (Tanswell et al, 1976). This could reflect the ionic strength sensitive sulfate binding site described by Scopes. However, a second sulfate binding with an apparent dissociation constant of 4 mM was also indicated from NMR studies (Tanswell et al, 1976) and this could be the site we have monitored and now refer to as the anion binding site (cf. Table 1).

Recktenwald and Hess (1977) have recently presented evidence for a regulatory site on yeast mitchondrial ATPase which will bind substrate and other anions, and the kinetic similarities between this ATPase and phosphoglycerate kinase are striking. The specificity of the ATPase regulatory site lies in the $H_mXO_4^{n-}$ structure, in particular it is accessible to anions such as SO_4^{2-} and HPO_4^{2-} with an X-O bond length of 1.45 Å. These workers also presented evidence that occupancy of the regulatory site influenced only the catalytic site located on the same protomer. Since phosphoglycerate kinase is a monomer, the regulatory site and catalytic center are located on a single polypeptide chain.

CHAPTER V

METAL ION BINDING TO PHOSPHOGLYCERATE KINASE

A. INTRODUCTION

In addition to the proposed involvement of the divalent metal ions in nucleotide substrate binding, kinetic work has suggested that certain of these metal ions bound to the enzyme at a non-catalytic site (see Chapter I, section F.3). During the study of anion binding to yeast phosphoglycerate kinase (Chapter IV) it was noted that Mg^{2+} increased the absorbance of phosphoglycerate kinase labelled with mercurial. Also, it was reasoned that the binding of Na^{+} introduced error in titrations of the labelled enzyme with the di- and monovalent anions, and that Mg^{2+} was able to prevent this through binding to the labelled enzyme (see Chapter IV, section C.1). Previous studies (Stinson, 1974) suggested that the rate of reaction between the enzyme and the mercurial could be employed to monitor Mg^{2+} binding. For reasons discussed in Chapter III, section A.2(b) the effect of ligands on the rate constant for the reaction between the enzyme and DTNB rather than the mercurial was determined.

The susceptibility of phosphoglycerate kinase to proteolytic inactivation by trypsin or proteinase A is increased when the kinase is modified with the mercurial or DTNB (see Chapter III, sections C.2(a) and C.2(b)). The effect of certain divalent metal ions on the susceptibility of the native and mercurial- or DTNB-modified kinase to proteolytic inactivation by these endopeptidases was also examined.

B. MATERIALS AND METHODS

1. Materials

General materials used are listed in Chapter II, section A. The following were obtained from Fisher Scientific (Fairlawn, N.J.): thioglycollic acid, $MgCl_2$, $MnCl_2$, $CaCl_2$, $CoCl_2$, CuCl, $CuCl_2$, $FeCl_3$, $ZnCl_2$, $ZnSO_4$, and Zn metal. $CdCl_2$ was purchased from Mallinckrodt (St. Louis, Mo.). The trisodium salt of citrate, the disodium salt of 3-phosphoglycerate, and the sodium salts of ADP and AMP were from Sigma Chemical Co. (St. Louis, Mo.). Analytical grade $NiCl_2$ was obtained from B.D.H. (Poole, England). β-Mercaptoethanol was a product of Eastman Kodak (Rochester, N.Y.).

For the spectrophotometric binding studies the buffer was 5.0 mM Tes adjusted to a final pH of 8.0 with sodium hydroxide. Proteolytic inactivation studies were buffered in 50 mM thriethanolamine adjusted to pH 7.5 with sodium hydroxide.

Stock solutions of metal ions were prepared in water with the exception of CuCl which was dissolved in 2 M hydrochloric acid. Stock solutions of Zn^{2+} were quantitated by atomic absorption and were kindly performed by the Department of Laboratory Medicine at the University of Alberta Hospital. Each dissociation constant between metal ion and enzyme was calculated on the assumption that all the metal ion added was available for binding.

2. Methods

a) Titrations of mercurial-labelled phosphoglycerate kinase with metal ions

General methods used are outlined in Chapter II, section B. Scatchard plots (Scatchard, 1950) were used to determine the dissociation constants and the number of metal ion binding sites per molecule of enzyme labelled with mercurial.

b) Rate of reaction between phosphoglycerate kinase and DTNB in the presence of metal ions

General methods used are outlined in Chapter II, section B.

Rate constants for the reaction between phosphoglycerate kinase and DTNB in the presence and absence of metal ions were calculated by the difference method described by Gutfreund (1965). Scatchard analysis (Scatchard, 1950) of the data gave the dissociation constants for the native enzyme and the metal ions and the number of metal ion binding sites. For the less tightly bound metal ions dissociation constants were obtained from plots of the reciprocal of the rate constant *vs.* the reciprocal of the metal ion concentration (Marshall and Cohen, 1977).

The second-order rate constant for the reaction between enzyme and DTNB varied with the concentration of the latter reagent (see Chapter III, section C.1(b). However, the dissociation constants for the metal ions did not change with DTNB concentration.

c) Inactivation of phosphoglycerate kinase by trypsin or proteinase A in the presence of metal ions

General methods used are outlined in Chapter II, section B.

The effect of Zn^{2+} on the susceptibility of native and mercurial- or DTNB-modified phosphoglycerate kinase to inactivation by trypsin or proteinase A was also examined. Experimental protocol was as described for inactivation of the modified kinase by trypsin or proteinase A (see Chapter II, section B.7). The effect of Mg^{2+} on trypsin inactivation of the native enzyme was carried out according to the procedure described for the native kinase (see Chapter II, section B.7). The effect of Zn^{2+} on the rate of hydrolysis of benzoyl arginine *p*-nitroanilide by trypsin was determined as described in Chapter II (section B.8)

C. RESULTS

1. Titrations of Mercurial-Labelled Phosphoglycerate Kinase with Metal Ions

Titration of the mercurial-labelled enzyme with each metal ion resulted in an increase in absorbance at 410 nm of the bound chromophore. A typical binding curve for Mg^{2+} is shown in figure 12A. Only a slight increase in absorbance occurred during metal ion titrations of a similar concentration of the mercurial bound to thioglycollic acid (data not shown). With the exception of Cd^{2+}, each metal ion showed similar maximum absorbance changes at equivalent enzyme-

FIGURE 12

Metal ion titrations of phosphoglycerate kinase labelled with mercurial. The mercurial-labelled enzyme (10.0 μM) was titrated with Mg^{2+} (●) or Zn^{2+} (▵) and the absorbance was measured at 410 nm. (A) Binding curves; (B) Scatchard plot of the Mg^{2+} data from (A). The fraction of the maximum change in absorbance obtained with each addition of metal ion is r, and the concentration of free metal ion is u. The titration procedure is described in Chapter II, section B.4. The buffer was 5.0 mM Tes, pH 8.0.

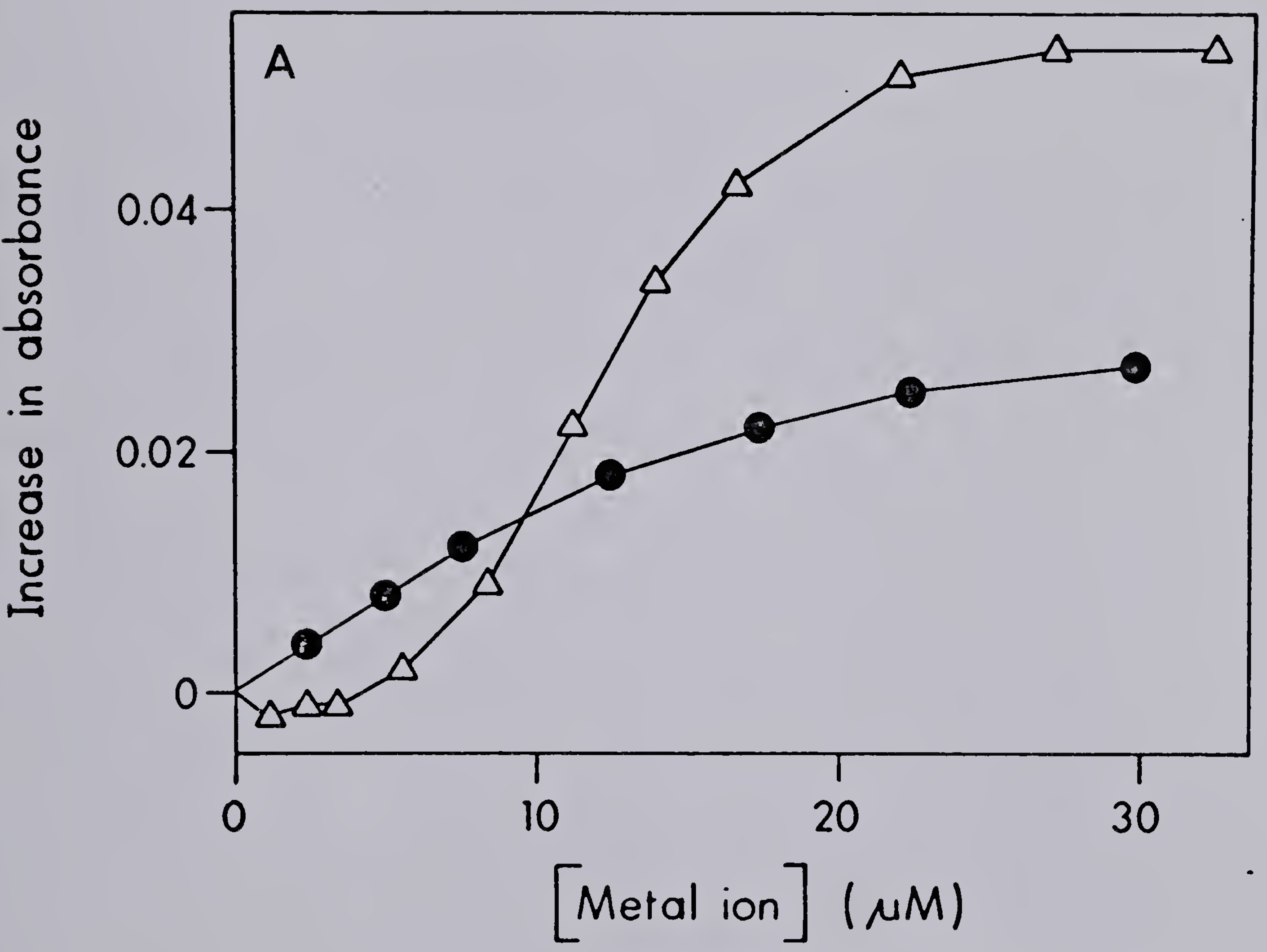
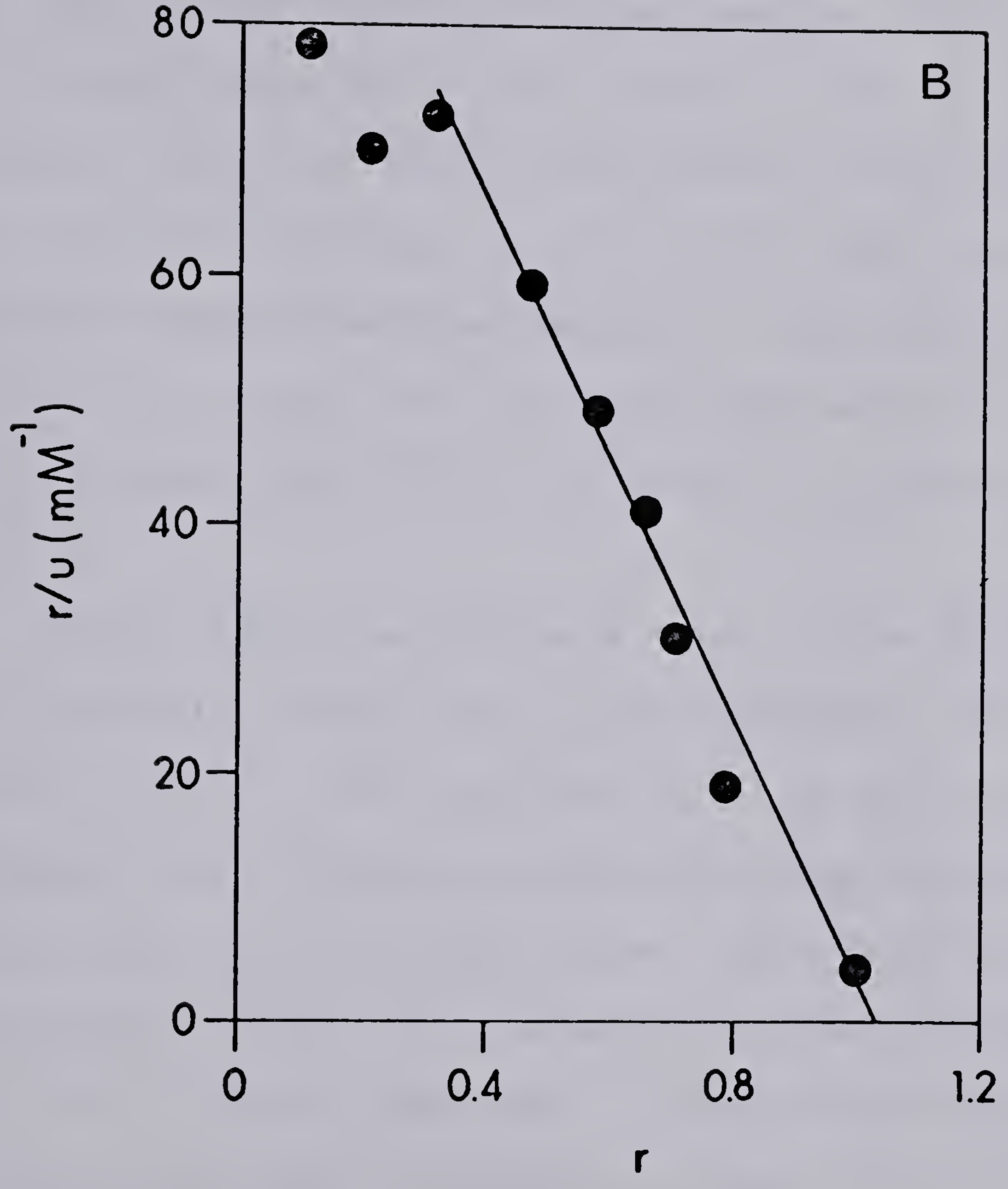

A
Increase in absorbance
0.04
0.02
0
0
10
20
30
[Metal ion] (μM)

FIGURE 12

Metal ion titrations of phosphoglycerate kinase labelled with mercurial. The mercurial-labelled enzyme (10.0 μM) was titrated with Mg^{2+} (●) or Zn^{2+} (△) and the absorbance was measured at 410 nm. (A) Binding curves; (B) Scatchard plot of the Mg^{2+} data from (A). The fraction of the maximum change in absorbance obtained with each addition of metal ion is r, and the concentration of free metal ion is u. The titration procedure is described in Chapter II, section B.4. The buffer was 5.0 mM Tes, pH 8.0.


B
r/υ (mM^{-1})
80
60
40
20
0
0
0.4
0.8
1.2
r

mercurial concentrations (Table 3); the absorbance change for Cd^{2+} was twice as high as with the other metal ions and this could suggest that Cd^{2+} competed with the mercurial for the enzyme's thiol.

Titrations with Zn^{2+} were unique in that an increase in absorbance did not occur until the concentration of Zn^{2+} approached that of the enzyme-mercurial complex (Fig. 12A). This lag increased with increasing concentration of the labelled enzyme (data not shown) and probably reflected relatively tight Zn^{2+} binding to a site which did not alter the environment of the bound chromophore. This site will be referred to as the 'tight' Zn^{2+} binding site and the dissociation constant for Zn^{2+} at this site is probably near to 10^{-8} M.

Figure 12B is a Scatchard plot of the Mg^{2+} binding data and is typical of those for all of the metal ions with the exception of Zn^{2+}. The Scatchard plot for Zn^{2+} was concave upwards and a binding constant for this metal ion was estimated from the titration curve. From Table 3 it can be seen that the dissociation constants for the divalent metal ions are of similar magnitude. Dissociation constants from duplicate titrations differed by no more than a factor of two. Also included in Table 3 are the number of binding sites obtained from Scatchard plots. For each metal ion examined this value was approximately one, indicating that there is only one metal ion binding site which can influence the environment of the bound chromophore. Although the number of Zn^{2+} binding sites was also one (Table 3), the dissociation constant for this metal ion estimated from the

TABLE 3

Binding Parameters for Metal Ions Determined from Titrations of Phosphoglycerate Kinase Labelled with Mercurial

The dissociation constant for Zn^{2+} was estimated from the response curve (Fig. 12A) and corrected for the enzyme concentration.

Metal ion	Dissociation constant	Metal ion bound to enzyme	Maximum chromogenicity increase
	μM	mol/47,000g	$\Delta A/\mu M$
Mn^{2+}	8	1.02	0.0042
Co^{2+}	10	0.84	0.0045
Mg^{2+}	18	0.96	0.0044
Ca^{2+}	28	1.03	0.0044
Cd^{2+}	43	1.02	0.0088
Zn^{2+}	8	0.94	0.0056

binding curve was unchanged in the presence of a Mn^{2+} concentration sufficient to saturate its own site. With the exception of Zn^{2+}, binding of all metal ions was mutually exclusive.

To further investigate the interaction between the metal ion binding site and the Zn^{2+} binding sites, the mercurial-labelled enzyme was titrated with Mg^{2+} or Mn^{2+} in the presence of two Zn^{2+} concentrations. The lower concentration of Zn^{2+} was sufficient to saturate its tight binding site and the higher concentration was sufficient to half-saturate its weaker site. Technical factors prevented using higher Zn^{2+} concentrations. The results of these titrations with Mg^{2+} are shown in figure 13. The dissociation constant for Mg^{2+} was not changed by either Zn^{2+} concentration, although the higher concentration did lower the spectral difference. This effect would be explained if Mg^{2+} can not bind to enzyme that has both Zn^{2+} sites occupied, in effect reducing the enzyme concentration by one-half. Alternatively, the chromogenic change that occurs when Mg^{2+} binds to the enzyme with 1 1/2 Zn^{2+} bound could be lower than the change occuring when Mg^{2+} binds to the enzyme with only 1 Zn^{2+} bound.

2. Rate of Reaction Between Phosphoglycerate Kinase and DTNB in the Presence of Metal Ions

Each of the divalent metal ions examined increased the rate constant for the reaction between native phosphoglycerate kinase and DTNB. The response curve for Co^{2+} is shown in figure 14A. Individual metal ions at a

FIGURE 13

The effect of Zn^{2+} on titrations of the phosphoglycerate kinase-mercurial complex with Mg^{2+}. Titrations of 10.0 μM of the labelled kinase were in the absence (●) and presence of Zn^{2+} at concentrations of 4.8 μM (▲) or 11.8 μM (■). The titration procedure is described in Chapter II, section B.4. The buffer was 5.0 mM Tes, pH 8.0.

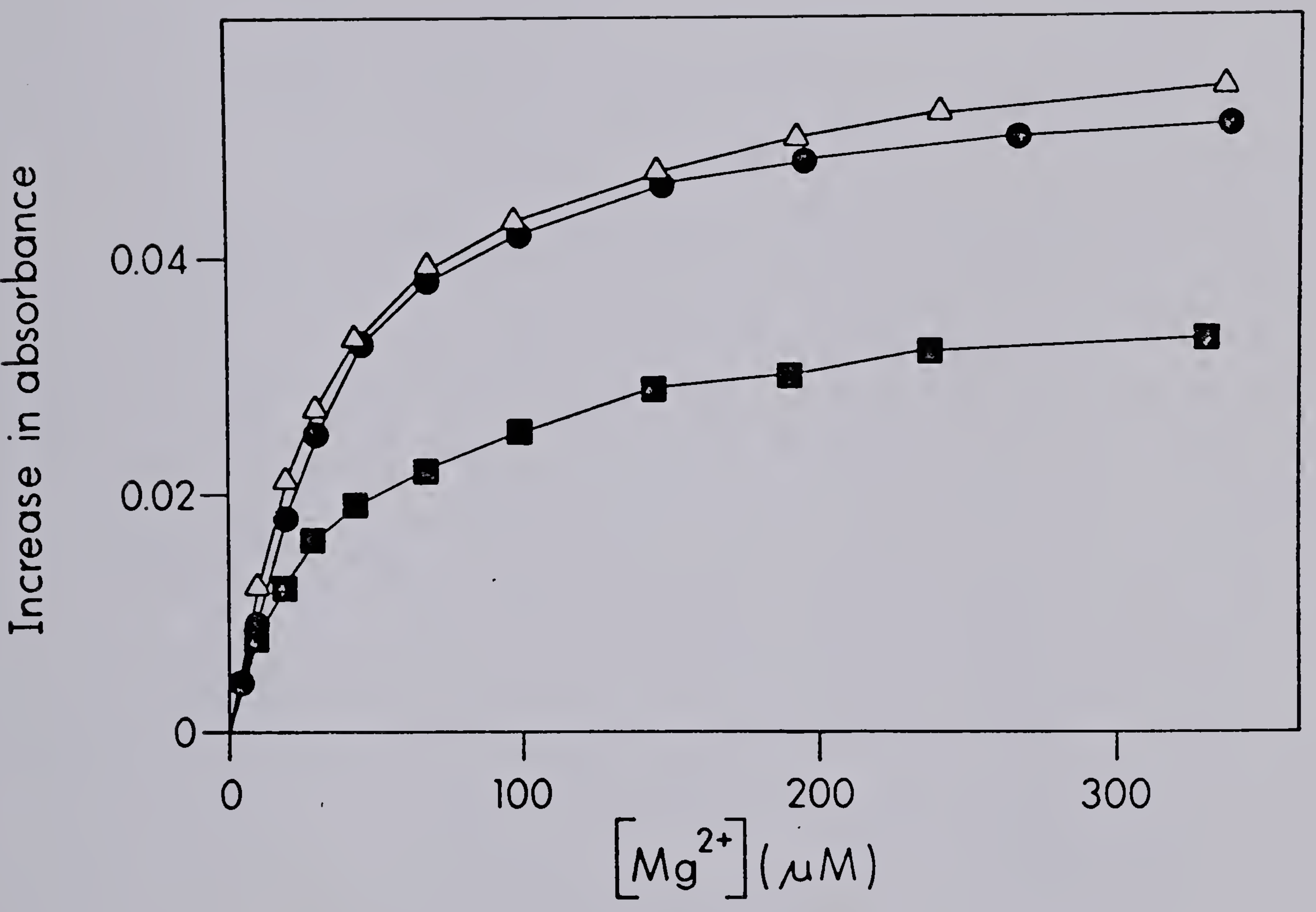

Increase in absorbance
0.04
0.02
0
0
100
200
300
$[Mg^{2+}]$ (μM)

FIGURE 14

The effect of Co^{2+} on the rate of reaction between phosphoglycerate kinase and DTNB. (A) Binding curve; (B) Scatchard plot of the data from (A). Rate constants, k, for the reaction between 5.0 μM enzyme and 0.1 mM DTNB were calculated as described in this chapter, section B.2(b). The fraction of the maximum change in rate constant obtained at each Co^{2+} concentration is r, and the concentration of free Co^{2+} is u. The experimental procedure is outlined in Chapter II, section B.5. The buffer was 5.0 mM Tes, pH 8.0.

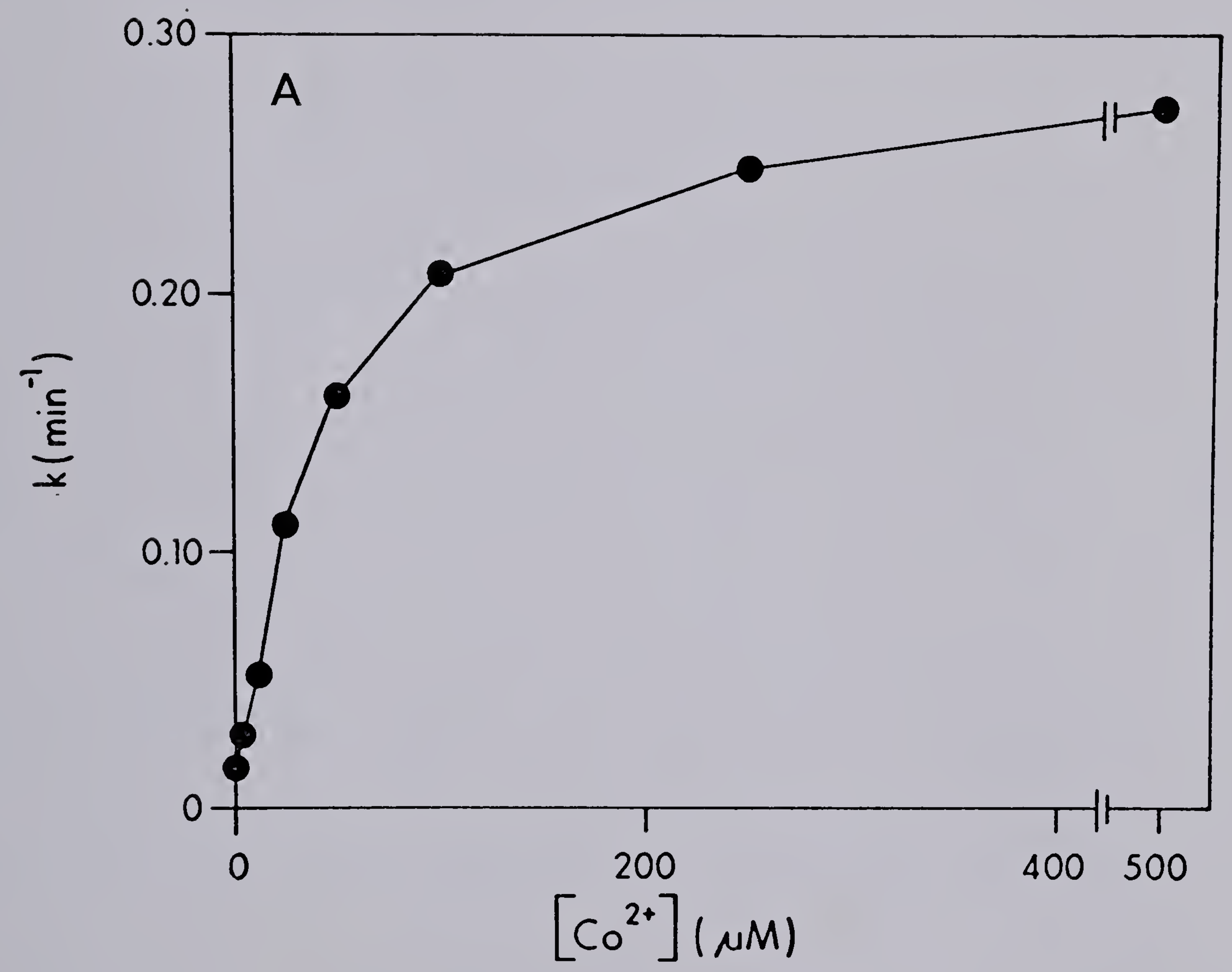
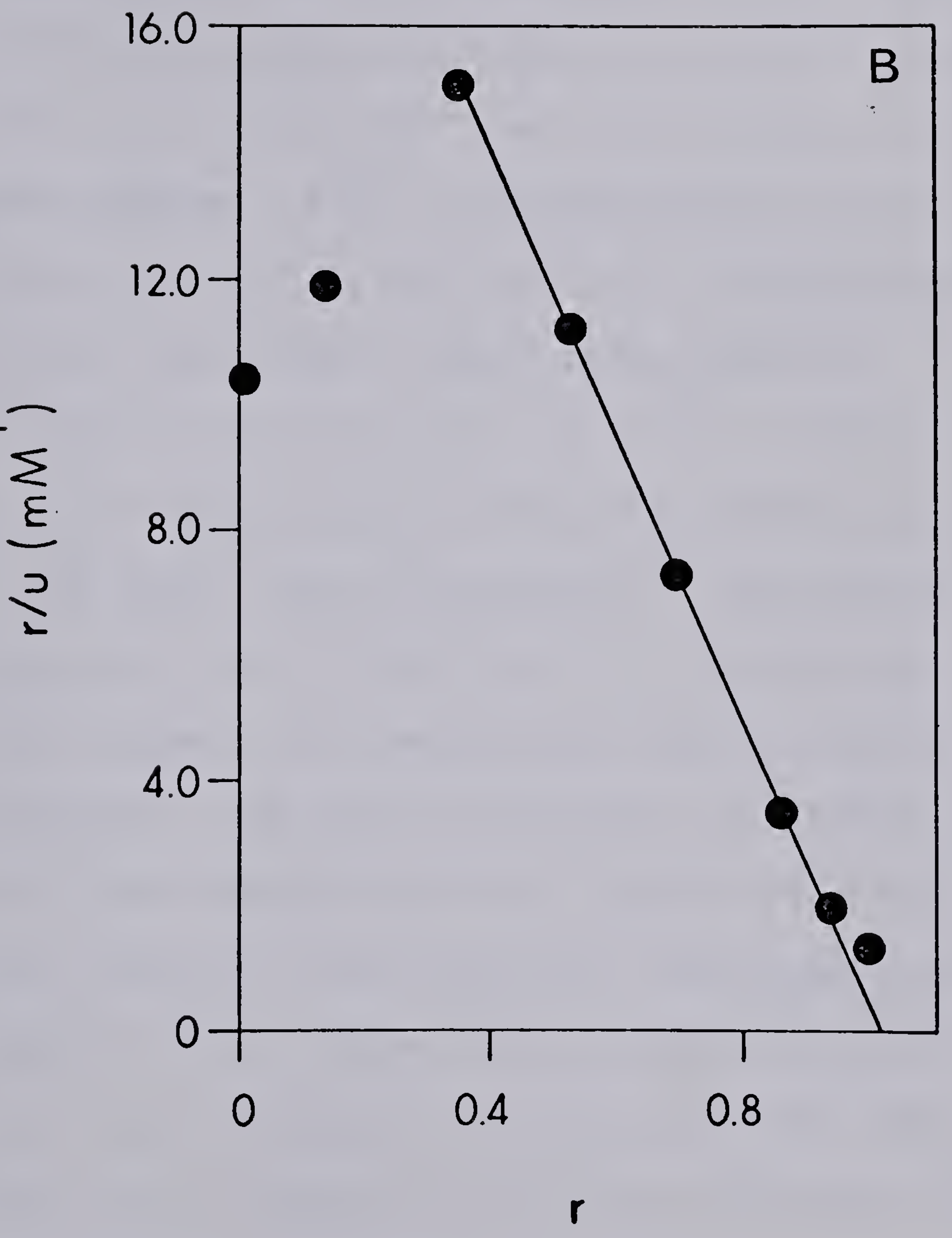

A
0.30
0.20
0.10
0
k (min^{-1})
0
200
400
500
[Co^{2+}] (μM)

FIGURE 14

The effect of Co^{2+} on the rate of reaction between phosphoglycerate kinase and DTNB. (A) Binding curve; (B) Scatchard plot of the data from (A). Rate constants, k, for the reaction between 5.0 μM enzyme and 0.1 mM DTNB were calculated as described in this chapter, section B.2(b). The fraction of the maximum change in rate constant obtained at each Co^{2+} concentration is r, and the concentration of free Co^{2+} is u. The experimental procedure is outlined in Chapter II, section B.5. The buffer was 5.0 mM Tes, pH 8.0.


B
16.0
12.0
8.0
4.0
0
r/u (mM^{-1})
0
0.4
0.8
r

concentration of approximately 100 μM did not change the spontaneous rate of DTNB hydrolysis. At a similar concentration most of the metal ions did not alter the absorbance spectra of DTNB, or the nitrothiophenolate anion. Cd^{2+}, Cu^{2+}, and Cu^{1+} decreased, in a time-dependent fashion, the absorbance of the released anion. This precluded determination of dissociation constants for the latter two ions but the reaction with Cd^{2+} was slow enough to allow such a determination. Fe^{3+} at concentrations up to 0.20 mM did not significantly increase the rate constant and probably did not bind to the site(s) under investigation.

A typical Scatchard plot for Co^{2+} is shown in figure 14B. The dissociation constants for the enzyme and certain of the metal ions, determined in this manner or from double reciprocal plots of the data, are summarized in Table 4. The values for the divalent metal ions are of similar magnitude, and agree with those determined from titrations of the enzyme-mercurial complex (Table 3). Dissociation constants from duplicate experiments agreed to within a factor of two. The number of binding sites obtained from the Scatchard plots indicate that there is only one metal ion binding site that can influence thiol availability to DTNB (Table 4)

Two aspects of the Zn^{2+} response curve were unique. Firstly, as noted with titrations of the enzyme-mercurial complex, an initial lag was apparent (Fig. 15), and this increased with increasing enzyme concentration (data not shown). Secondly, the maximum rate constant in the presence

TABLE 4

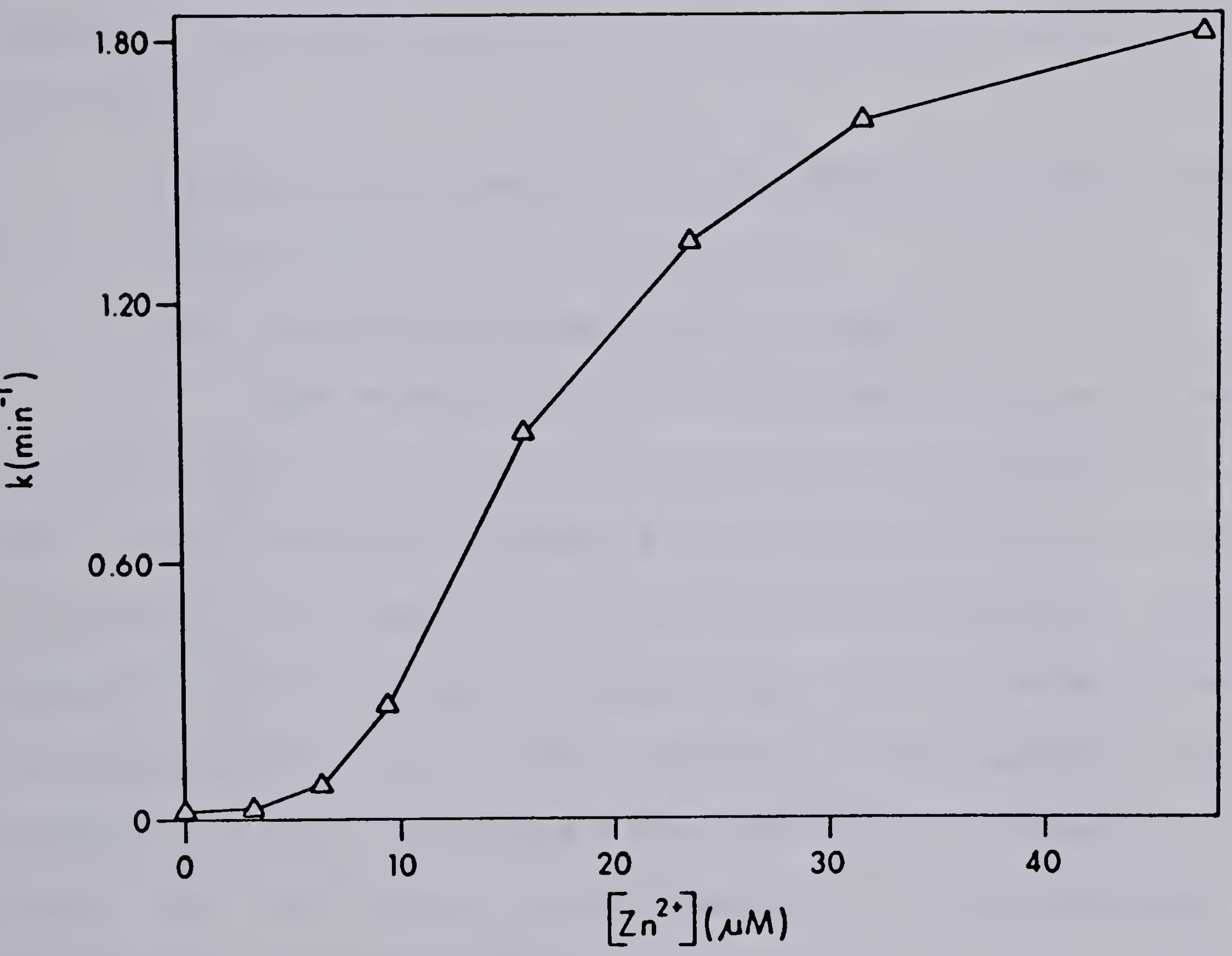
Binding Parameters for Metal Ions Determined from the Increase in Rate Constant for the Reaction Between Phosphoglycerate Kinase and DTNB

Values omitted could not be accurately determined from Scatchard plots and the dissociation constants for these metal ions were determined from double reciprocal plots. The dissociation constant for Zn^{2+} was estimated from the response curve (Fig. 15) and corrected for enzyme concentration.

Metal ion	Dissociation constant	Metal ion bound to enzyme
	μM	mol/47,000 g
Mn^{2+}	4	1.09
Co^{2+}	44	1.02
Mg^{2+}	31	-
Ca^{2+}	120	1.14
Cd^{2+}	48	-
Ni^{2+}	62	-
Zn^{2+}	10	1.22

FIGURE 15

The effect of Zn^{2+} on the rate of reaction between phosphoglycerate kinase and DTNB. The rate constant, k, for the reaction between 5.0 μM enzyme and 0.2 mM DTNB was calculated as described in this chapter, section B.2(b). The experimental procedure is outlined in Chapter II, section B.5. The buffer was 5.0 mM Tes, pH 8.0.


1.80
1.20
0.60
0
k(min^{-1})
0
10
20
30
40
[Zn^{2+}](μM)

of Zn^{2+} was up to 6 times higher at the same concentration of DTNB than that observed with most of the other divalent metal ions. Saturation of the metal ion binding site with Mn^{2+} failed to alter either the initial lag or the high rate constants characteristic of the Zn^{2+} response curve, and the estimated dissociation constant for Zn^{2+} as its weak site was unchanged. Furthermore, the presence of Zn^{2+} at a concentration equal to its estimated dissociation constant (Table 4) did not alter the affinity of the enzyme for Mg^{2+}.

3. Proteolytic Inactivation of Phosphoglycerate Kinase in the Presence of Metal Ions

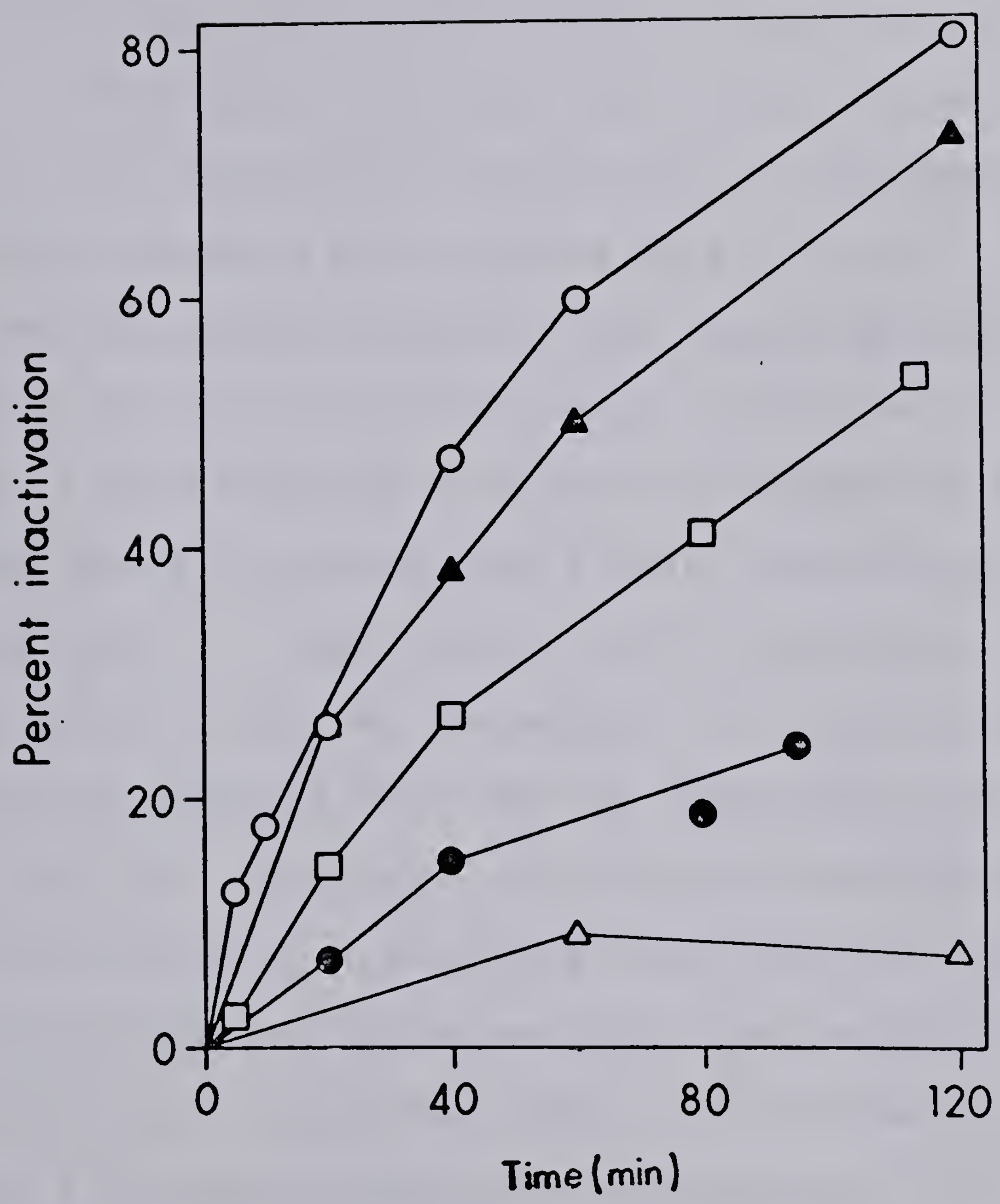
a) Native phosphoglycerate kinase

The susceptibility of phosphoglycerate kinase to inactivation by trypsin is increased in the presence of Zn^{2+}. At a phosphoglycerate kinase-to-trypsin molar ratio of 500 to 1 the activity of the kinase decreased by less than 10% after 120 min of incubation. At the same kinase-to-trypsin ratio and in the presence of 52.5 μM Zn^{2+} the kinase was 80% inactivated after 120 min of incubation (Fig. 16). The effect of Zn^{2+} varied in a dose-related manner and approached a maximum at 52.5 μM. The concentration of Zn^{2+} estimated to give the half-maximal increase in rate of inactivation was 20 μM, assuming a single binding site.

Zn^{2+} also increased the susceptibility of phosphoglycerate kinase to inactivation by proteinase A. At a kinase-to-proteinase A molar ratio of 9.1 to 1 the

FIGURE 16

The effect of Zn^{2+} on the rate of inactivation of phosphoglycerate kinase by trypsin. The Zn^{2+} concentrations were 15.8 μM (●), 26.2 μM (□), 39.4 μM (▲), and 52.5 μM (○). The rate of inactivation of the kinase in the absence of Zn^{2+} is also shown (△). The incubation mixtures contained 2.0 μM phosphoglycerate kinase and were prepared as described in Chapter II, section B.7. The kinase-to-trypsin molar ratio was 500 to 1. Aliquots were taken at the times indicated following the addition of trypsin and assayed as described in Chapter II, section B.1. Activity is expressed as percent inactivation relative to the activity determined prior to the addition of trypsin. The buffer was 50 mM triethanolamine, pH 7.5.


80
60
40
20
0
Percent inactivation
0
40
80
120
Time (min)

kinase activity decreased by less than 3% after 60 min of incubation. At the same kinase-to-proteinase A ratio and in the presence of 52.5 μM Zn^{2+} the rate of inactivation was initially rapid followed by a slower rate; after 5 and 90 min of incubation 19.0 and 58.3% inactivation, respectively, had occurred (Fig. 17). Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate indicated that in the presence of 52.5 μM Zn^{2+} proteolytic degradation of phosphoglycerate kinase by proteinase A had occurred (Fig. 18, gel 3). The apparent molecular weights of the resulting polypeptides were similar to those detected during incubation of proteinase A with the native or mercurial-labelled kinase at the same kinase-to-proteinase A molar ratio (Fig. 6, gels 2,4,5, and 6). Approximately 80% inactivation of phosphoglycerate kinase by proteinase A in the presence of Zn^{2+} had occurred at the time the sample was electrophoresed (Fig. 18, gel 3) but the relative proportions of the polypeptides were different from those detected when the mercurial-labelled kinase was 80% inactivated by proteinase A (Fig. 6, gel 6). After more than 6 h of incubation with proteinase A the major endproduct of proteolysis of the mercurial-labelled kinase was a polypeptide with a molecular weight of 28,000 (Fig. 18, gel 4). But in the presence of Zn^{2+} and after a similar incubation time the major product from native phosphoglycerate kinase was a polypeptide with a molecular weight of 36,000 (Fig. 18, gel 3).

FIGURE 17

The effect of Zn^{2+} on the rate of inactivation of native and mercurial-labelled phosphoglycerate kinase by proteinase A. The open symbols represent the native kinase and the closed symbols represent the mercurial-labelled kinase; (○) and (●) indicate the rate of inactivation in the absence of Zn^{2+}, and (□) and (■) indicates the rate of inactivation in the presence of 52.5 μM Zn^{2+}. The incubation mixtures contained 2.0 μM phosphoglycerate kinase and were prepared as described in Chapter II, section B.7. The kinase-to-proteinase A molar ration was 9.1 to 1. Aliquots were taken at the times indicated following the addition of proteinase A and assayed as described in Chapter II, section B.1. Activity is expressed as percent inactivation relative to the activity determined prior to the addition of proteinase A. The buffer was 50 mM triethanolamine, pH 7.5.

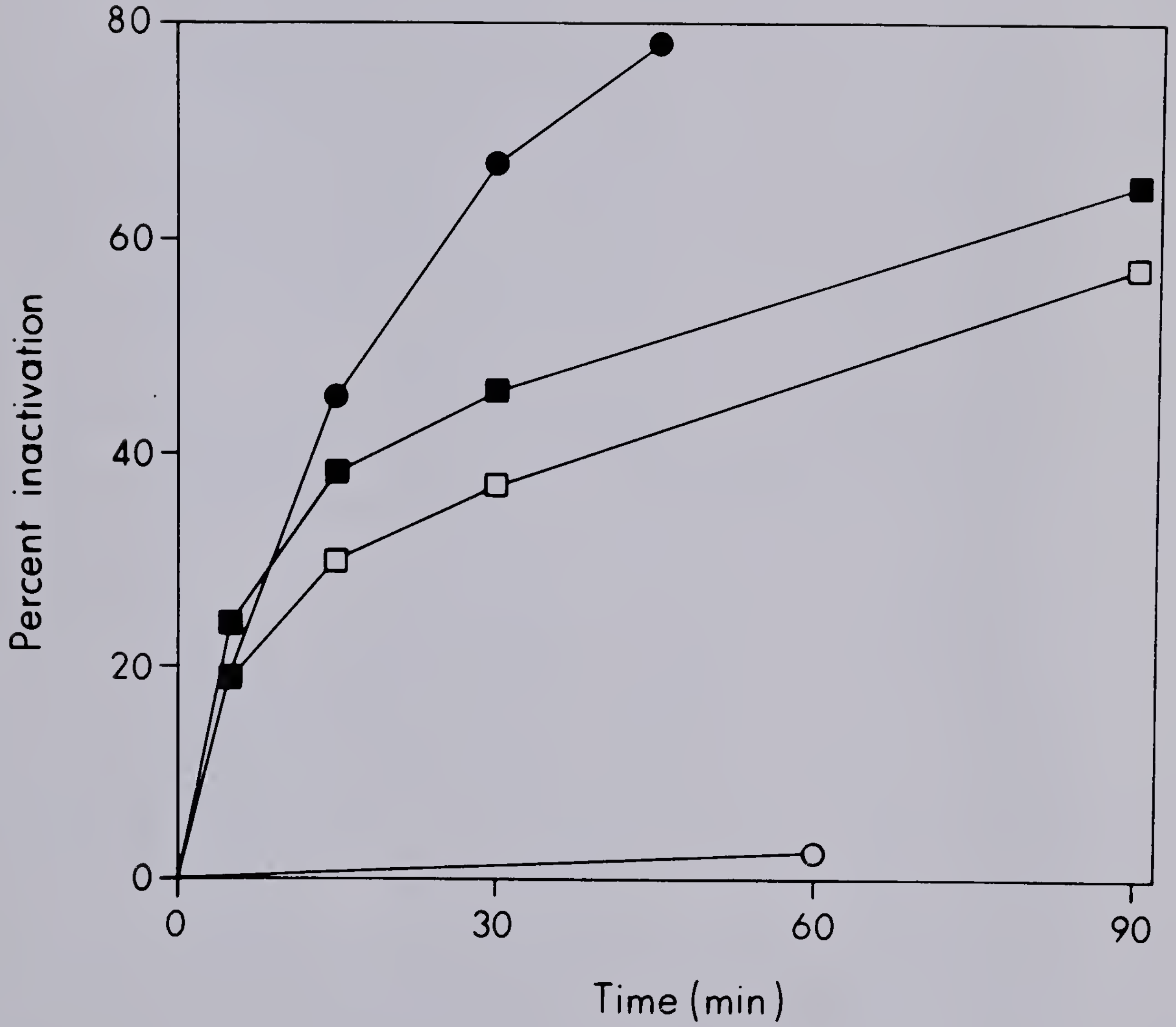
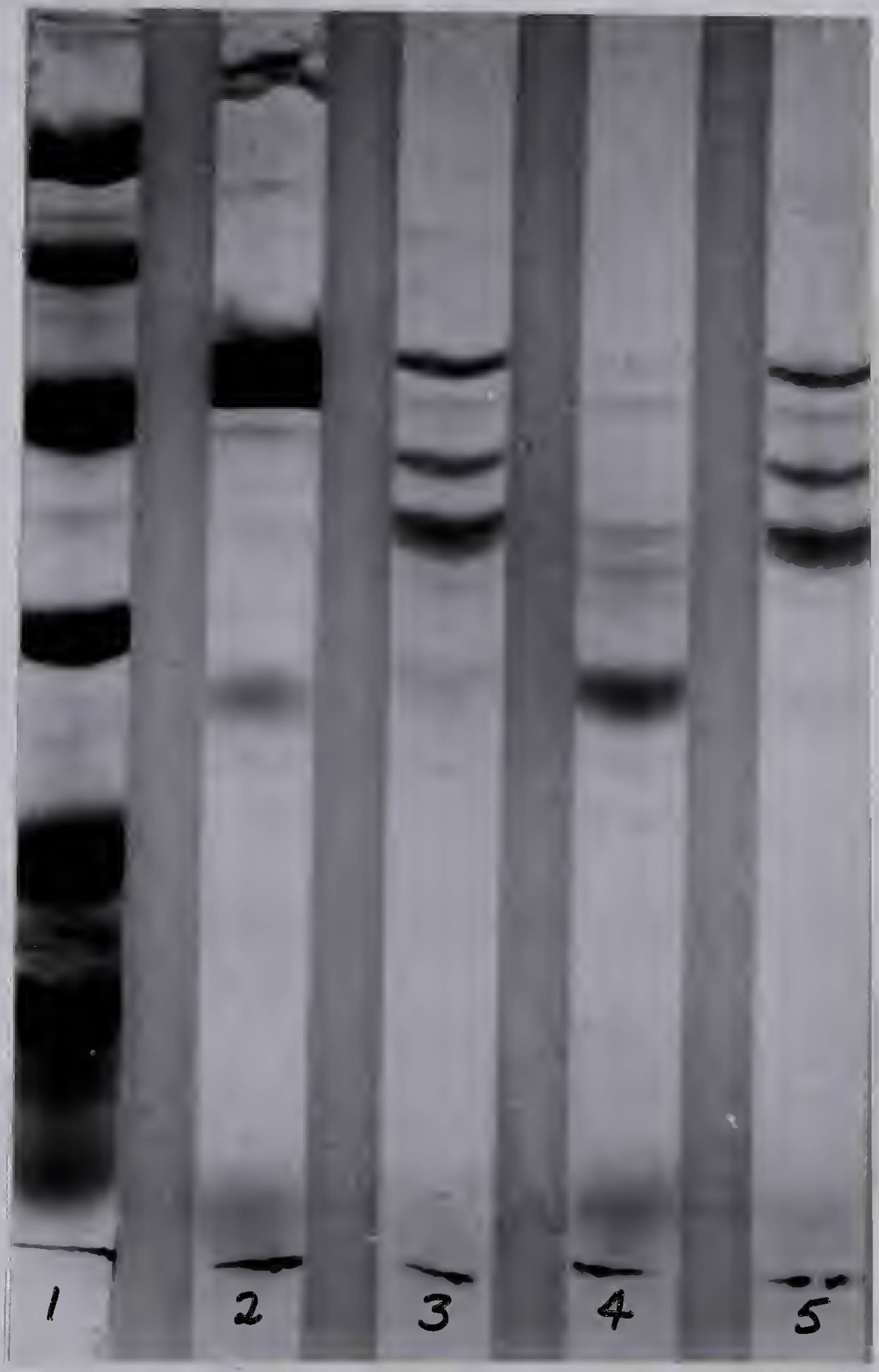

80
60
40
20
0
0
30
60
90
Percent inactivation
Time (min)

FIGURE 18

The effect of Zn^{2+} on the peptides formed during limited proteinase A digestion of native and mercurial-labelled phosphoglycerate kinase. Incubation mixtures were prepared as described in Chapter II, section B.7 and the kinase-to-proteinase A molar ratio was 9.1 to 1. Electrophoresis and protein staining were according to the methods outlined in Chapter II, section B.9. Gel 2 shows the native phosphoglycerate kinase (shown in figure 6, gel 2 at 120 min) after ~8 h incubation with proteinase A. For gels 3,4, and 5, the kinase was incubated with proteinase A as in figure 17. Gel 3 shows the native kinase which was incubated in the presence of 52.5 μM Zn^{2+} and frozen following ~9 h of incubation. Gel 4 shows the mercurial-labelled kinase which was incubated in the absence of Zn^{2+} and frozen following ~6 h of incubation. Gel 5 shows the mercurial-labelled kinase which was incubated in the presence of 52.5 μM Zn^{2+} and frozen following ~8 h of incubation. An aliquot of each thawed sample was prepared for electrophoresis as described in Chapter II, section B.9. Gel 1 shows the subunit molecular weight markers which are (top to bottom): phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, soybean trypsin inhibitor, and lysozyme (see legend to Fig. 4).


1
2
3
4
5

In the absence of either endopeptidase, the activity of phosphoglycerate kinase in the presence of 52.5 μM Zn^{2+} decreased by less than 11% during a 120 min incubation. At a concentration of 17.4 μM Zn^{2+} did not change the rate of hydrolysis of benzoyl arginine *p*-nitroanilide by trypsin.

b) Mercurial-or DTNB-modified phosphoglycerate kinase

Zn^{2+} at a concentration of 52.5 μM did not alter the susceptibility of mercurial-or DTNB-modified phosphoglycerate kinase to inactivation by trypsin (data not shown). However, figure 17 shows that 52.5 μM Zn^{2+} did decrease the susceptibility of the mercurial-labelled kinase to inactivation by proteinase A such that the rate of inactivation was similar to that observed with native phosphoglycerate kinase in the presence of Zn^{2+} (see this chapter, section C.3(a)).

The results of polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulfate suggested that in the presence of Zn^{2+}, inactivation of the labelled kinase by proteinase A was proteolytic (Fig. 18, gel 5) and gave rise to polypeptides of similar mobilities to those which were detected during incubation of proteinase A with the native or mercurial-labelled kinase. However, as discussed for the native kinase (see this chapter, section C.3(a)) the relative proportions of the polypeptides differ when the mercurial-labelled kinase was approximately 80% inactivated in the absence (Fig. 6, gel 6) and presence of

Zn^{2+} (Fig. 18, gel 5). After a similar length of incubation of the native or labelled kinase in the presence of Zn^{2+}, the relative proportions of the polypeptides were the same (Fig. 18, gels 3 and 5). After prolonged incubation of the native or labelled kinase the major polypeptide had a molecular of 28,000 but after a similar length of incubation in the presence of Zn^{2+} a polypeptide with a molecular weight of 36,000 was the major component.

D. DISCUSSION

Each of the divalent metal ions caused a similar increase in the absorbance of the enzyme-mercurial complex (Table 3) or, with the exception of Zn^{2+}, in the availability of the enzyme's single thiol for reaction with DTNB. These changes were of comparable magnitude but directionally opposite to those which occur when the anions bind (see Chapter IV, sections C.1 and C.2). Scatchard analysis of the data (Figs. 12B and 14B, Tables 3 and 4) indicates that the degree of occupancy of a single metal ion binding site influences the environment of the cysteinyl residue. With the exception of Zn^{2+}, binding of the metal ions was mutually exclusive and this suggests that the metal ions other than Zn^{2+} bind to the same site. Metal ion titrations caused only a slight increase in absorbance of thioglycollic acid or β-mercaptoethanol complexed with the mercurial, and failed to increase the spontaneous rate of DTNB hydrolysis or, with the exception of Cd^{2+}, to change the absorbance spectra of DTNB or its nitrothiophenolate anion. These

findings suggest that the metal ions bind to phosphoglycerate kinase and thereby alter the environment of the enzyme's thiol.

The maximum rate constant for the reaction between phosphoglycerate kinase and 0.2 mM DTNB was six times greater in the presence of Zn^{2+} compared to that observed in the presence of most of the other divalent metal ions. This difference, and the lag in the Zn^{2+} response curves apparent in both detection systems (Figs. 12A and 15) were repeatedly demonstrated with Zn^{2+} prepared from $ZnCl_2$, $ZnSO_4$, or Zn metal. Thus it is unlikely that a contaminant could account for these findings.

The results of this study indicate that occupancy of the tight Zn^{2+} binding site (see this chapter, section C.1) does not change the affinity of the mercurial-labelled enzyme for other metal ions (Fig. 13), and saturation of the metal ion binding site does not prevent subsequent binding of Zn^{2+} (data not shown). At concentrations of Zn^{2+} compatible with occupancy of the weak Zn^{2+} binding site, the susceptibility of phosphoglycerate kinase to inactivation by trypsin is increased (Fig. 16) while the other metal ions do not change this parameter. These findings suggest that the two Zn^{2+} binding sites are distinct from the metal ion binding site.

A correlation does not exist between the dissociation constants for the metal ions (excluding Zn^{2+}) reported here (Tables 3 and 4) and their crystal ionic radii as listed by Phipps (1976). Some of the variation in affinity of the

metal ions other than Zn^{2+} could be due to differences in the availability of each metal ion for binding under the experimental conditions. Dissociation constants for the metal ions with the exception of Zn^{2+} are lower than those previously determined by others. Larsson-Raźnikiewicz (1970b) reported that the uncompetitive inhibitor constants for Mg^{2+} and Mn^{2+} were approximately 3 mM and greater than 1.0 mM, respectively. However, the buffer used for the kinetic and binding experiments by Larsson-Raźnikiewicz (1970b), *tris*hydroxymethylaminomethane, could lead to falsely high inhibitor or dissociation constants because of its ability to chelate metal ions (see for example Allen et al, 1967). Chapman et al (1974) reported a dissociation constant for Mn^{2+} of 0.5 to 1.0 mM but it is unclear from their report whether the enzyme was dialyzed prior to use; our results indicated that Mg^{2+} prevents Na^+ binding to the enzyme (see Chapter IV, section C.1) and high levels of other cations, such as NH_4^+, could weaken Mn^{2+} binding.

The presence of a high affinity metal ion binding site on yeast phosphoglycerate kinase could account for the findings by Roustan et al (1973) that the dissociation constants for the nucleotides are unchanged in the presence of metal ions. From examination of their figures it appears that an absorbance maximum at 278 nm correlates with binding of Mg^{2+} to the enzyme. This could indicate that the enzyme can compete with the nucleotides for Mg^{2+} such that binding of the free nucleotides is monitored even in the presence of the metal ion.

The dissociation constant estimated from the spectrophotometric binding studies for the weaker Zn^{2+} binding site (Tables 3 and 4) is similar to the uncompetitive inhibitor constant of approximately 20 μM reported for Zn^{2+} with respect to Zn-ATP (Larsson-Raźnikiewicz, 1970b). However, it is possible that saturation of the tight Zn^{2+} binding site is important in terms of the strong inhibition of catalysis that is characteristic of this metal ion. Based on the results of this study it is difficult to draw conclusions as to the relationship of the two Zn^{2+} binding sites. We could, for example, be monitoring chemically equivalent sites which show negative cooperativity of binding and are located such that occupancy of the first site does not alter the environment of the enzyme's thiol while occupancy of the second site does. Alternatively, we could be monitoring two sites which differ in their affinity for Zn^{2+} and are located such that the tight site does not alter the thiol's environment while occupancy of the weak site does; a slight overlap during titration of these two sites could lead to the apparent S-shaped curves characteristic of Zn^{2+} binding (Figs. 12A and 15). Scatchard plots of the Zn^{2+} data from the mercurial or DTNB system showed apparent positive cooperativity (data not shown). A Hill plot (see for example Segel, 1975) and a similar plot described by Whitehead (1978) indicated that negative cooperativity occurred between the two Zn^{2+} binding sites (data not shown). However, it is important to note that the sensitivity of the two spectrophotometric methods

used here is such that an enzyme concentration of 5 to 10 μM is required. This is close to the estimated dissociation constant for Zn^{2+} at its weak site (Tables 3 and 4) and clearly does not meet the requirements for determination of an accurate dissociation constant or interaction coefficient when the fraction of bound ligand is monitored.

It is unlikely that the metal ions, including Zn^{2+} (at its weak binding site), bind directly to the single thiol of yeast phosphoglycerate kinase. The absorbance increase observed in titrations of the enzyme-mercurial complex with each metal ion was too small to be accounted for by dissociation of the mercurial from the enzyme. Direct binding of metal ions to the thiol should result in blocking rather than facilitation of the thiol for reaction with DTNB. When one considers the experimental error associated with the two spectrophotometric probes, the dissociation constants determined by each method agree (Tables 3 and 4) despite the fact that the metal ion binding is monitored pricr to modification of the enzyme in the DTNB system or subsequent to labelling of the enzyme with mercurial. Furthermore, rate constant plots of the reaction between phosphoglycerate kinase and DTNB in the presence of metal ions were linear. These findings suggest that occupancy of the metal ion binding site, or the weak Zn^{2+} binding site, alter the environment of the cysteinyl residue through conformational changes. These results do not, however, allow us to draw conclusions as to the mechanism(s) responsible for the changes in the absorbance of the mercurial or reactivity of the enzyme's thiol with DTNB. The initial lag apparent in the response curves determined using the

mercurial or DTNB system was similar at the same concentration of mercurial-labelled or native enzyme. This finding suggests that Zn^{2+} also binds with similar affinity to its tight (see this chapter, section C.1) binding site present on either the labelled or native enzyme.

In 1974, Friedberg reviewed the effects of metal ion binding on protein structure and concluded that, in general, divalent metal ions such as Ca^{2+} are ligated by carbonyl and carboxyl oxygens while Zn^{2+} binding is mediated primarily by imidazole groups, sometimes in combination with carboxyl functions. Similar conclusions have been made in reviews by Blundell and Jenkins (1977) and Sarkar (1977). The dissociation constants estimated here for both Zn^{2+} binding sites of yeast phosphoglycerate kinase fall within the range of 10^{-5} to 10^{-8} M reported for most of the Zn^{2+} binding proteins (Friedberg, 1974). Also, the tightness of Zn^{2+} binding reported here is similar to that obtained with chemically synthesized complexes which contain two or three imidazole groups (Tang et al, 1978). A recent structural study of yeast phosphoglycerate kinase (Fattoum et al, 1978) places seven of its eight histidyl residues on the largest cyanogen bromide cleavage fraction which could constitute the thiol-containing lobe B (see Chapter I, section D.3). It is possible that one of the Zn^{2+} binding sites reported here corresponds to one of the heavy metal binding sites indicated from X-ray crystallographic studies to be at or near the enzyme's single thiol (see Chapter I, section D.3) and this could be the same as the Gd^{3+} binding site detected

by Tanswell et al (1976).

CHAPTER VI

INTERACTION BETWEEN ANION AND METAL ION BINDING TO PHOSPHOGLYCERATE KINASE

A. INTRODUCTION

In the two preceeding chapters experimental evidence was presented that yeast phosphoglycerate kinase contains one anion, one metal ion, and two Zn^{2+} binding sites. Since each of these ligands have been shown by others to exert regulatory effects on the catalytic activity of the enzyme (see Chapter IV, section D and Chapter I, section F.3). the simultaneous binding of anions and metal ions was monitored. This was accomplished by determining the composite effect of anions and metal ions on the rate constant for the reaction between phosphoglycerate kinase and DTNB.

B. MATERIALS AND METHODS

General materials used are listed in Chapter II, section A. Anions and metal ions used for these studies are listed in Chapter IV, section B.1 and Chapter V, section B.1, respectively.

General methods used are outlined in Chapter II, section B. Concentrations of the ligands were chosen such that a significant amount of anion-metal ion complex did not occur and these calculations were based on the following dissociation constants; Mg-3-phosphoglycerate, 10.5 mM (Larsson-Raźnikiewicz, 1972); Ca-ADP, 0.25 mM (O'Sullivan and Perrin, 1964); Zn-AMP, 4.0 mM (Sigel et al, 1967).

C. RESULTS

The effect of 3-phosphoglycerate, in the presence or absence of Mg^{2+}, on the rate constant for the reaction between phosphoglycerate kinase and DTNB is shown in figure 19. In the absence of Mg^{2+} a dissociation constant for 3-phosphoglycerate of 9.3 μM is taken from the intercept on the abscissa (see Chapter IV, section B.2) and this value is unaffected by the presence of 50 μM or 1.0 mM $MgCl_2$. Also, up to 20 μM 3-phosphoglycerate does not change the enzyme's affinity for Mg^{2+} (data not shown). Similar findings were obtained with ADP and Ca^{2+} in that the dissociation constant for ADP was unchanged in the presence of Ca^{2+} and ADP did not change the enzyme's affinity for Ca^{2+}.

AMP at a concentration of 1.0 mM (K_d=0.5 mM see Chapter IV, section C.2) did not change the length of the initial lag of the Zn^{2+} response curve nor did it prevent Zn^{2+} concentrations up to 28.9 μM from increasing thiol availability for reaction with DTNB (Fig. 20). At these Zn^{2+} concentrations the affinity of the enzyme for AMP was unchanged (data not shown). A complete Zn^{2+} response curve in the presence of AMP could not be obtained as significant chelation of the metal ion by AMP would occur.

D. DISCUSSION

The results of these experiments indicate that the anion and metal ion binding sites (Fig. 19), and anion and tight Zn^{2+} binding sites (Fig. 20) do not interact in terms of binding affinities.

FIGURE 19

The effect of 3-phosphoglycerate, in the presence and absence of Mg^{2+}, on the rate of reaction between phosphoglycerate kinase and DTNB. Mg^{2+} was absent (○) or present at concentrations of 50 μM (▲) or 1.0 mM (□). The rate constant, k, for the reaction between 10.1 μM enzyme and 1.0 mM DTNB was calculated as described in Chapter IV, section B.2(b). The experimental procedure is outlined in Chapter II, section B.5. The buffer was 5.0 mM Tes, pH 8.0.

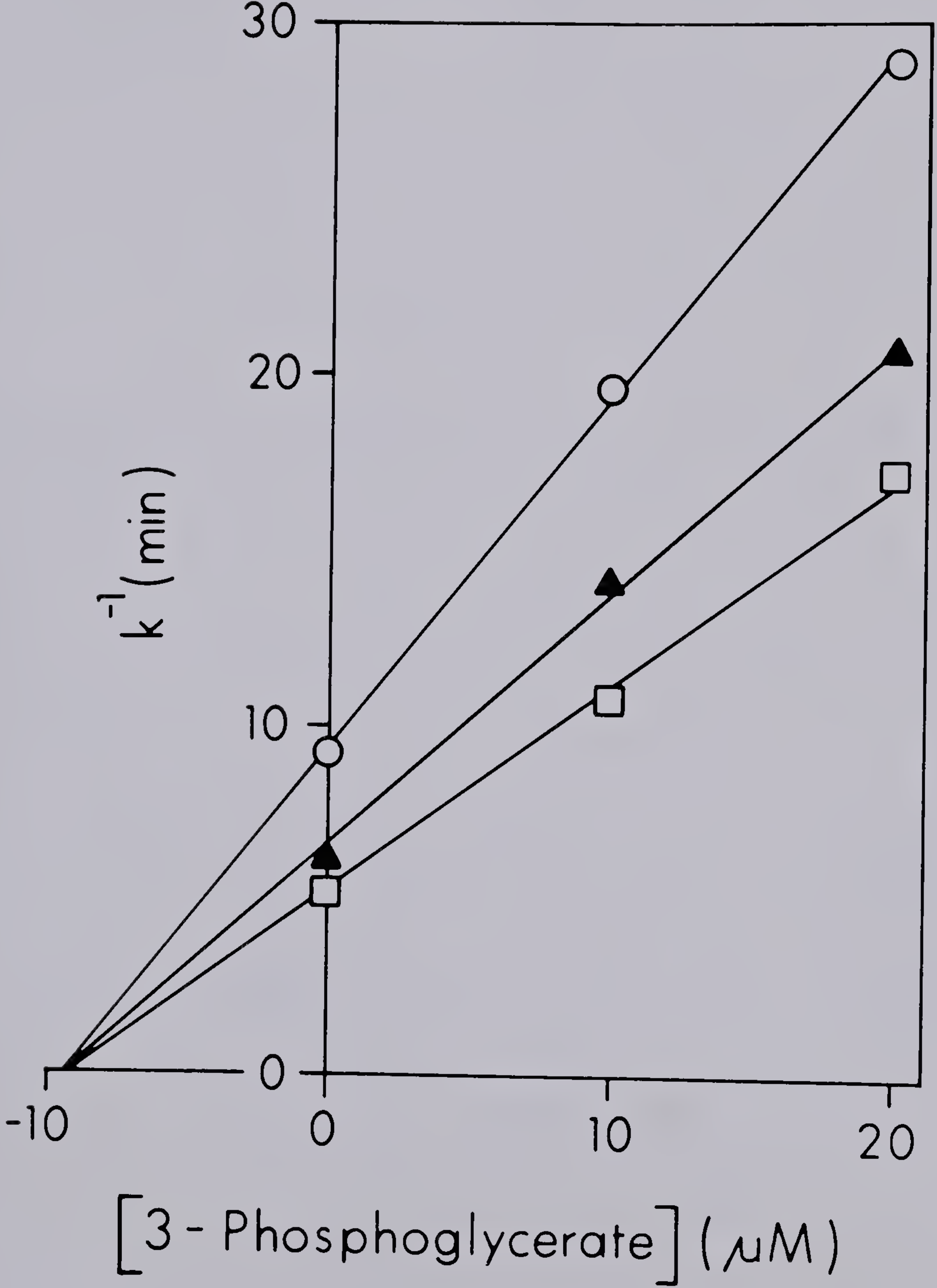
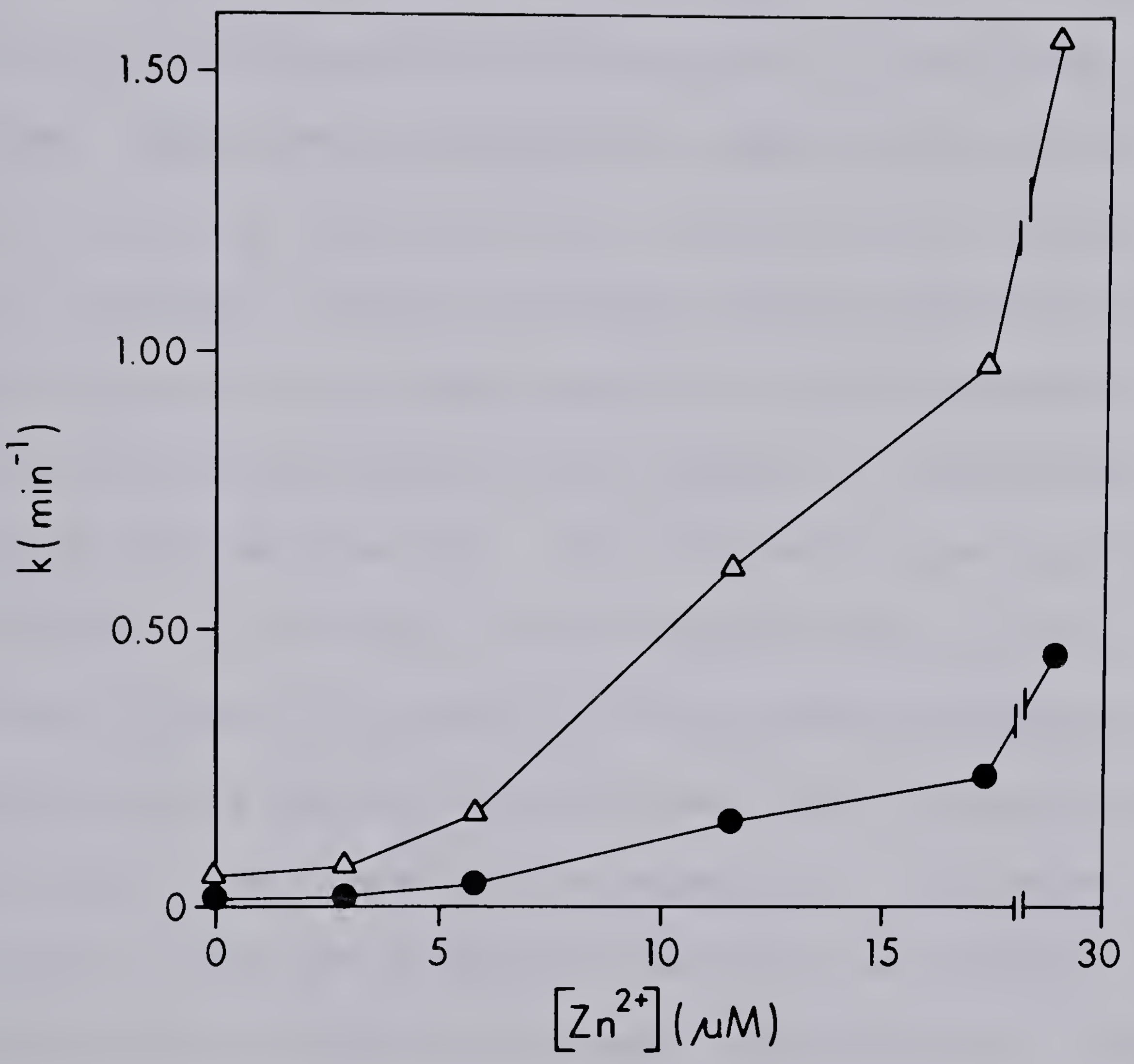

30
20
10
0
k^{-1} (min)
-10
0
10
20
[3 - Phosphoglycerate] (μM)

FIGURE 20

The effect of Zn^{2+}, in the presence and absence of AMP, on the rate of reaction between phosphoglycerate kinase and DTNB. AMP was absent (△) or present at a concentration of 1.0 mM (●). The rate constant, k, for the reaction between 5.0 μM enzyme and 0.2 mM DTNB was calculated as described in Chapter V, section B.2(b). The experimental procedure is outlined in Chapter II, section B.5. The buffer was 5.0 mM Tes, pH 8.0.


1.50
1.00
0.50
0
k (min^{-1})
0
5
10
15
30
[Zn^{2+}] (μM)

CHAPTER VII

GENERAL DISCUSSION

This thesis presents evidence obtained from binding studies using two spectrophotometric probes that substrate and non-substrate anions bind to yeast phosphoglycerate kinase. Each anion decreased the absorbance of 2-chloromercuri-4-nitrophenol bound to the single thiol of the enzyme and also decreased the reactivity of the thiol toward DTNB. The results indicated that binding of the substrate anions to the catalytic center was not being monitored. Several lines of evidence suggested that the two spectrophotometric probes monitored conformational changes which occurred when anions bound to the enzyme (see Chapter IV, section D). The divalent anion succinate was also shown to decrease the susceptibility of phosphoglycerate kinase to inactivation by bovine pancreatic trypsin or yeast proteinase A and this effect was observed at succinate concentrations compatible with occupancy of the anion binding site. Thus it is likely that the rate of proteolytic inactivation monitors the same conformational changes as those detected with the spectrophotometric probes.

Metal ion binding studies using two spectrophotometric probes also indicated that divalent metal ions bind to yeast phosphoglycerate kinase. Each metal ion increased the absorbance of the mercurial bound to the enzyme's single thiol and increased the reactivity of the thiol toward DTNB. Two Zn^{2+} binding sites were indicated and these sites

differed in their affinity for Zn^{2+}. Both Zn^{2+} binding sites appeared to be distinct from the site which bound each of the other metal ions studied. The results indicated that the two spectrophotometric probes were insensitive to Zn^{2+} binding at its tight site but monitored conformational changes which occurred when Zn^{2+} bound to its weaker site, and when the other metal ions bound to the metal ion binding site. Zn^{2+} was also shown to increase the susceptibility of phosphoglycerate kinase to proteolytic inactivation by bovine pancreatic trypsin or yeast proteinase A, and this effect was detected at concentrations of Zn^{2+} compatible with binding to the weak site. The other metal ions did not alter the rate of kinase inactivation by trypsin. These findings likely indicate that the proteolytic inactivation studies monitor the same conformational changes which are detected by the spectrophotometric probes when Zn^{2+} binds to its weak site.

The binding studies reported here suggest that anions, metal ions, or Zn^{2+} bind to phosphoglycerate kinase and, in doing so, cause the enzyme to undergo conformational changes. The specificities of these ligand binding sites correlates with the specificities of regulatory sites indicated from kinetic work by others. Occupancy of the anion binding site could for example, account for reports of activation by substrates (see for example Larsson-Raźnikiewicz and Schierbeck, 1977) and non-substrate anions (Larsson-Raźnikiewicz and Jansson, 1973; Scopes, 1976; Scopes, 1978a). Similarly, the dissociation constant reported here for Zn^{2+} at its weak binding site is in good

agreement with the uncompetitive inhibitor constant for Zn^{2+} with respect to Zn-ATP (Larsson-Raźnikiewicz, 1970b; Cavell and Scopes, 1976). The results of the binding studies reported here indicate that the anion and Zn^{2+} binding sites do not interact in terms of binding affinities. If these sites do possess regulatory properties then their occupancy could simultaneously influence catalytic activity.

In addition to providing a means whereby the binding of anions and Zn^{2+} to phosphoglycerate kinase can be monitored, the results of the proteolytic inactivation studies reported here (see above) could be of potential physiological importance. According to Goldberg and Dice (1974) the enzymes responsible for intracellular degradation of proteins probably act in a similar fashion to the well-characterized proteinases used *in vitro*, thus one can correlate the relative resistance to proteolysis *in vitro* with the *in vivo* degradative rate. These authors conclude that the selectivity of degradation is primarily a function of protein conformation. Assuming that the anion and Zn^{2+} binding sites are present under *in vivo* conditions and retain their properties as described here, the degree of occupancy of each site could simultaneously influence the intracellular degradative rate of phosphoglycerate kinase. If assignment of the regulatory properties to these binding sites is correct then succinate binding should put the kinase into a conformation which is more active and less susceptible to proteolysis, while the binding of Zn^{2+} should cause the kinase to take on a

conformation which is less active and more susceptible to proteolysis. Tanabe et al (1977) have reported that the *in vitro* susceptibility of acetyl coenzyme A carboxylase to proteolysis by lysosomal extracts is diminished by citrate, an activator of the enzyme, and is enhanced by palmityl-coenzyme A, an inhibitor of the enzyme.

In addition to studying the effect of anions and Zn^{2+} on the susceptibility of phosphoglycerate kinase to inactivation by trypsin or proteinase A, the effect of the spectrophotometric probes on the rate of inactivation was also examined. Modification of the single thiol of yeast phosphoglycerate kinase with the mercurial or DTNB caused a marked increase in the susceptibility of the kinase to proteolytic inactivation *in vitro*. This finding is significant since the susceptibility to proteolytic inactivation could be a useful probe to examine the role of this non-essential thiol in the maintenance of the native enzyme's conformation. Whether this observation is of potential physiological importance is uncertain since the changes could reflect the properties of the modifying reagents. Modification of the thiol or saturation of the weak Zn^{2+} binding site cause a similar increase in the rate of phosphoglycerate kinase inactivation by trypsin but these effects were not additive. Based on the results of the spectrophotometric binding studies, modification of the thiol does not prevent Zn^{2+} binding. These findings could suggest that each treatment causes a similar conformational change.

BIBLIOGRAPHY

Achilles, W., Cumme, G.A. & Hoppe, H. (1972) 2,3-Diphosphoglycerate: Acid Dissociation and Complex Formation with Magnesium, Potassium, and Sodium. Determination of Acidity and Stability Constants by pH Titration. Acta Biol. Med. Germ. 29, 531-538.

Ali, M. & Brownstone, Y.S. (1976) A Study of Phosphoglycerate Kinase in Human Erythrocytes II. Kinetic Properties. Biochim. Biophys. Acta, 445, 89-103.

Allen, D.E., Baker, D.J. & Gillard, R.D. (1967) Metal Complexing by Tris Buffer. Nature, 214, 906-907.

Allen, R.C., Harley, R.A. & Talamo, R.C. (1974) A New Method for Determination of Alpha-1-antitrypsin Phenotypes Using Isoelectric Focusing on Polyacrylamide Gel Slabs. Am. J. Clin. Pathol. 62, 732-739.

Arese, P., Bosia, A., Pescarmona, G.P. & Till, U. (1974) 2,3-Diphosphoglycerate Synthesis by Human White Ghosts. FEBS Lett. 49, 33-36.

Arese, P., Bosia, A., Pescarmona, G.P. & Till, U. (1977) The Role of Red Cell Membrane in the Regulation of Glycolysis and the 2,3-Bisphosphoglycerate-Cycle. Acta Biol. Med. Germ. 36, 481-490.

Arvidsson, L. & Larsson-Raźnikiewicz, M. (1973) The SH Group of Yeast Phosphoglycerate Kinase. Biochim. Biophys. Acta, 310, 430-436.

Arvidsson, L., Schierbeck, B. & Larsson-Raźnikiewicz, M. (1976) Characterization of Charge Isomers of Yeast Phosphoglycerate Kinase. Evidence for Intracellular Differences. Acta Chem. Scand. [B] 30, 228-234.

Bacharach, A.D.E., Markland, F.S., Pellino, A. & Weber, B.H. (1977) Modification of Yeast 3-Phosphoglycerate Kinase: Isolation and Sequence Determination of a Nitrated Active-Site Peptide and Isolation of a Carboxyl Modified Active-Site Peptide. Biochem. Biophys. Res. Commun. 74, 165-171.

Barman, T.E. (ed.)(1969) *Enzyme Handbook*, Vol. II, pp. 615, 618, Springer-Verlag Berlin, Heidelberg.

Bartlett, G.R. (1958) Organization of Red Cell Glycolytic Enzymes: Cell Coat Phosphorus Transfer. Ann. N.Y. Acad. Sci. 75, 110-114.

Blake, C.C.F. & Evans, P.R. (1974) Structure of Horse Muscle Phosphoglycerate Kinase. Some Results on the Chain Conformation, Substrate Binding and Evolution of the Molecule from a 3 Å Fourier Map. J. Mol. Biol. 84, 585-601.

Blake, C.C.F., Evans, P.R. & Scopes, R.K. (1972) Structure of Horse-Muscle Phosphoglycerate Kinase at 6 Å Resolution. Nature, 235, 195-198.

Blundell, T.L. & Jenkins, J.A. (1977) The Binding of Heavy Metals to Proteins. Chemical Society Reviews, 6, 139-171.

Bojanovski, M., Kulbe, K.D., & Lamprecht, W. (1974) Liver 3-Phosphoglycerate Kinase. Purification and Some Molecular Properties of the Bovine-Liver Enzyme. Eur. J. Biochem. 45, 321-331.

Brake, A.J. & Weber, B.H. (1974) Evidence for an Essential Carboxyl Group for Yeast Phosphoglycerate Kinase. Reaction with Woodward's Reagent K. J. Biol. Chem. 249, 5452-5457.

Brevet, A., Roustan, C., Desvages, G., Pradel, L.-A. & van Thoai, N. (1973) Yeast 3-Phosphoglycerate Kinase: Evidence for a Glutamyl Residue in the Phosphoryl Transfer. Eur. J. Biochem. 39, 141-147.

Bryant, T.N., Watson, H.C. & Wendell, P.L. (1974) Structure of Yeast Phosphoglycerate Kinase. Nature, 247, 14-17.

Bücher, T. (1947) Über ein Phosphatürbertragendes Gärungsferment. Biochim. Biophys. Acta, 1, 292-314.

Cam A.E., & Cooper, D.W. (1978) Autosomal Inheritance of Phosphoglycerate Kinase in the Domestic Chicken (*Gallus domesticus*). Biochem. Genet. 16, 261-270.

Cavell, S. & Scopes, R.K. (1976) Isolation and Characterisation of the 'Photosynthetic' Phosphoglycerate Kinase from *Beta vulgaris*. Eur. J. Biochem. 63, 483-490.

Chapman, B.E., O'Sullivan, W.J., Scopes, R.K. & Reed, G.H. (1974) Binding of Mn-ADP^- to Phosphoglycerate Kinase. FEBS Lett. 41, 189-191.

Chapman, B.E., O'Sullivan, W.J., Scopes, R.K. & Reed, G.H. (1977) Magnetic Resonance Studies on Manganese-Nucleotide Complexes of Phosphoglycerate Kinase. Biochemistry, 16, 1005-1010.

Chapman, V.M. & Shows, T.B. (1976) Somatic Cell Genetic Evidence for X-Chromosome Linkage of Three Enzymes in the Mouse. Nature, 259, 665-667.

Chen, S.-H. & Giblett, E.R. (1972) Phosphoglycerate Kinase: Additional Variants and Their Geographic Distribution. Am. J. Hum. Genetics, 24, 229-230.

Chen, S.-H., Donahue, R.P., & Scott, C.R. (1976) Characterization of Phosphoglycerate Kinase from Human Spermatozoa. Fertil. Steril. 27, 699-701.

Chillar, R.K. & Beutler, E. (1976) Explanation for the Apparent Lack of Ouabain Inhibition of Pyruvate Production in Hemolysates: The "Backward" PGK Reaction. Blood, 47, 507-512.

Clarke, F.M. & Masters, C.J. (1975) On the Association of Glycolytic Enzymes with Structural Proteins of Skeletal Muscle. Biochim. Biophys. Acta, 381, 37-46.

Collier, H.B. (1973) A Note on the Molar Absorptivity of Reduced Ellman's Reagent, 3-Carboxylato-4-Nitrothiophenolate. Anal. Biochem. 56, 310-311.

Cooper, D.W., Vende Berg, J.L., Sharman, G.B. & Poole, W.E. (1971) Phosphoglycerate Kinase Polymorphism in Kangaroos provides Further Evidence for Paternal X Inactivation. Nature, 230, 155-157.

Cottreau, D., Kahn, A. & Boivin, P. (1976) Human Granulocyte Phosphoglycerate Kinase. Purification by Double Affinity Elution and Immunological Study. Enzyme, 21, 427-435.

De, B.K.& Kirtley, M.E. (1977) Interaction of Phosphoglycerate Kinase with Human Erythrocyte Membranes. J. Biol. Chem. 252, 6715-6720.

Duchon, G. & Collier, H.B. (1971) Enzyme Activities of Human Erythrocyte Ghosts: Effects of Various Treatments. J. Membrane Biol. 6, 138-157.

Ellman, G.L. (1959) Tissue Sulfhydryl Groups. Arch. Biochem. Biophys. 82, 70-77.

Erlanger, B.F., Kokowsky, N. & Cohen, W. (1961) The Preparation and Properties of Two New Chromogenic Substrates of Trypsin. Arch. Biochem. Biophys. 95, 271-278.

Fairbanks, G., Steck, T.L. & Wallach, D.F.H. (1971) Electrophoretic Analysis of the Major Polypeptides of the Human Erythrocyte Membrane. Biochemistry, 10, 2606-2617.

Fattoum, A., Roustan, C., Feinberg, J., Desvages, G. & Pradel, L.-A. (1978) Structural Studies on Yeast 3-Phosphoglycerate Kinase. Isolation by Affinity Chromatography and Characterization of the Peptides Produced by Cyanogen Bromide Cleavage. Location of the Single Cysteinyl Residue in the Primary Structure. Eur. J. Biochem. 82, 161-167.

Fifis, T. & Scopes, R.K. (1978) Purification of 3-Phosphoglycerate Kinase from Diverse Sources by Affinity Elution Chromatography. Biochem. J. 175, 311-319.

Fossel, E.T. & Solomon, A.K. (1978) Ouabain-Sensitive Interaction between Human Red Cell Membrane and Glycolytic Enzyme Complex in Cytosol. Biochim. Biophys. Acta, 510, 99-111.

Friedberg, F. (1974) Effects of Metal Binding on Protein Structure. Quarterly Reviews of Biophysics, 7, 1-33.

Goldberg, A.L. & Dice, J.F. (1974) Intracellular Protein Degradation in Mammalian and Bacterial Cells. Ann. Rev. Biochem. 43, 835-869.

Gupta, S.K. & Rothstein, M. (1976a) A Heat-Stable Factor which Aggregates 3-Phosphoglycerate Kinase from *Turbatrix aceti*. Biochem. Biophys. Res. Commun. 69, 48-54.

Gupta, S.K. & Rothstein, M. (1976b) Phosphoglycerate Kinase from Young and Old *Turbatrix aceti*. Biochim. Biophys. Acta, 445, 632-644.

Gutfreund, H. (1965) *An Introduction to the Study of Enzymes*, pp. 12-17, Blackwell Scientific Publications, Oxford and Edinburgh.

Gutfreund, H. & McMurray, C.H. (1970) Stoicheiometric, Reactivity and Environmental Probes for Sulfhydryl Groups in Enzymes. In: *Chemical Reactivity and Biological Role of Functional Groups in Enzymes* (Smellie, R.M.S. ed), pp. 39-47, Academic Press, London and New York.

Hata, T., Hayashi, R. & Doi, E. (1967) Purification of Yeast Proteinases Part III. Isolation and Physicochemical Properties of Yeast Proteinase A and C. Agr. Biol. Chem. 31, 357-367.

Heinz, F., Freimüller, B. & Obermeyer, J. (1974) Renal Phosphoglycerate Kinase. Hoppe Seylers Z. Physiol. Chem. 355, 1405-1414.

Hjelmgren, T., Arvidsson, L. & Larsson-Raźnikiewicz, M. (1976) Spectrophotometric pH Titrations and Nitration with Tetranitromethane of the Tyrosyl Residues in Yeast Phosphoglycerate Kinase. Biochim. Biophys. Acta, 445, 342-349.

Hjelmgren, T., Strid, L. & Arvidsson, L. (1976) An Essential Arginyl Residue in Phosphoglycerate Kinase from Yeast. FEBS Lett. 68, 137-140.

Janson, C.A. & Cleland, W.W. (1974) The Inhibition of Acetate, Pyruvate, and 3-Phosphoglycerate Kinases by Chromium Adenosine Triphosphate. J. Biol. Chem. 249, 2567-2571.

Johnson, P.E., Abbott, S.J., Orr, G.A., Sémériva, M. & Knowles, J.R. (1975) On the "Phosphoryl-Enzyme" of Phosphoglycerate Kinase. Biochem. Biophys. Res. Commun. 62, 382-389.

Johnson, P.E., Abbott, S.J., Orr, G.A., Sémériva, M. & Knowles, J.R. (1976) The "Phosphoryl-Enzyme" from Phosphoglycerate Kinase. Biochemistry, 15, 2893-2899.

Jörnvall, H. (1975) Acetylation of Protein N-terminal Amino Groups. Structural Observations on α-Amino Acetylated Proteins. J. Theor. Biol. 55, 1-12.

Kahn, A., Cottreau, D., Galand, C. & Boivin, P. (1976) Human Erythrocyte Phosphoglycerate Kinase Deficiency: Presence in a Deficient Patient of a Stable Variant with Lowered Catalytic Activity. Clin. Chim. Acta, 69, 21-28.

Kuntz, G.W.K., Eber, S., Kessler, W., Krietsch, H. & Krietsch, W.K.G. (1978) Isolation of Phosphoglycerate Kinases by Affinity Chromatography. Eur. J. Biochem. 85, 493-501.

Krietsch, W.K.G. & Bücher, T. (1970) 3-Phosphoglycerate Kinase from Rabbit Skeletal Muscle and Yeast. Eur. J. Biochem. 17, 568-580.

Krietsch, W.K.G. & Kuntz, G.W.K. (1978) Specific Enzymatic Determination of Adenosine Triphosphate. Anal. Biochem. 90, 829-831.

Krietsch, W.K.G., Krietsch, H., Kaiser, W., Dünnwald, M., Kuntz, G.W.K., Duhm, J. & Bücher, T. (1977) Hereditary Deficiency of Phosphoglycerate Kinase: A New Variant in Erythrocytes and Leucocytes, Not Associated with Haomolytic Anaemia. Eur. J. Clin. Invest. 7, 427-435.

Lambert, S.M. & Watters, J.I. (1957) The Complexes of Magnesium Ion with Pyrophosphate and Triphosphate Ions. J. Am. Chem. Soc. 79, 5606-5608.

Larsson-Raźnikiewicz, M. (1964) Kinetic Studies on the Reaction Catalyzed by Phosphoglycerate Kinase I. The Effect of Mg^{2+} and Adenosine 5'-Triphosphate. Biochim. Biophys. Acta, 85, 60-68.

Larsson-Raźnikiewicz, M. (1967) Kinetic Studies on the Reaction Catalyzed by Phosphoglycerate Kinase II. The Kinetic Relationships between 3-Phosphoglycerate, $Mg\text{-}ATP^{2-}$ and Activating Metal Ion. Biochim. Biophys. Acta, 132, 33-40.

Larsson-Raźnikiewicz, M. (1970a) Electrophoretic Purification as well as Some Physical and Chemical Characterizations of Phosphoglycerate Kinase from Yeast. Eur. J. Biochem. 15, 574-580.

Larsson-Raźnikiewicz, M. (1970b) The Phosphoglycerate Kinase Reaction and Its Metal Ion Specificity. Eur. J. Biochem. 17, 183-192.

Larsson-Raźnikiewicz, M. (1972) Complex Formation between 3-Phospho-D-glyceric Acid and Divalent Metal Ions. Eur. J. Biochem. 30, 579-583.

Larsson-Raźnikiewicz, M. (1973) Graphical Analyses on Binding of Ligands to a Two-Sited System. Theoretical Treatments Exemplified on Yeast Phosphoglycerate Kinase. Arch. Biochem. Biophys. 158, 754-762.

Larsson-Raźnikiewicz, M. & Arvidsson, L. (1971) Inhibition of Phosphoglycerate Kinase by Products and Product Homologues. Eur. J. Biochem. 22, 506-512.

Larsson-Raźnikiewicz, M. & Jansson, J.R. (1973) Activation of Yeast Phosphoglycerate Kinase by Salts of Monovalent Cations. FEBS Lett. 29, 345-347.

Larsson-Raźnikiewicz, M. & Malmström, B.G. (1961) The Metal-Ion Activation of 3-Phosphoglycerate Kinase in Correlation with Metal-Binding Studies. Arch. Biochem. Biophys. 92, 94-99.

Larsson-Raźnikiewicz, M. & Schierbeck, B. (1974) Studies on a Possible Phosphoryl-Enzyme Intermediate in the Catalytic Reaction of Yeast Phosphoglycerate Kinase. Biochem. Biophys. Res. Commun. 57, 627-634.

Larsson-Raźnikiewicz, M. & Schierbeck, B. (1977) The Possibility that the Substrates of Phosphoglycerate Kinase may Specifically Regulate the Direction of its Reversible Reaction. Biochem. Soc. Trans. 5, 770-771.

Malmström, B.G. & Larsson-Raźnikiewicz, M. (1962) 3-Phosphoglycerate Kinase. In: *The Enzymes* (Boyer, P., Lardy, H. and Myrback, K. eds.) Vol. 6, pp. 85-94, Academic Press, New York, N.Y.

Markland, F.S., Bacharach, A.D.E., Weber, B.H., O'Grady, T.C., Saunders, G.C. & Umemura, N. (1975) Chemical Modification of Yeast 3-Phosphoglycerate Kinase. J. Biol. Chem. 250, 1301-1310.

Marshall, M. & Cohen, P.P.(1977) Kinetics and Equilibrium of the Inactivation of Ornithine Transcarbamylases by Pyridoxal 5'-Phosphate. J. Biol. Chem. 252, 4276-4286.

Masters, C.J. & Holmes, R.S. (1975) *Haemoglobin, Isoenzymes and Tissue Differentiation*, pp. 95-97, North-Holland, Amsterdam.

McMurray, C.H. & Trentham, D.R. (1969) A New Class of Chromophoric Organomercurials and their Reactions with D-Glyceraldehyde 3-Phosphate Dehydrogenase. Biochem. J. 115, 913-921.

Melnick, R.L. & Hultin, H.O. (1973) On the Existence of a Complex of Glycolytic Enzymes. Bioenergetics, 5, 107-117.

Meyer, M.C. & Westhead, E.W. (1976) Interaction of Sulfate Ion with a Critical Tyrosine Residue in Yeast Phosphoglycerate Kinase Detected through the Tetranitromethane Reaction. FEBS Lett. 71, 25-28.

Mowbray, J. & Moses, V. (1976) The Tentative Identification in *Escherishia coli* of a Multienzyme Complex with Glycolytic Activity. Eur. J. Biochem. 66, 25-36.

Negelein, E. (1957) Synthesis, Determination, Analysis, and Properties of 1,3-Diphosphoglyceric Acid. In: *Methods in Enzymology* (Colowick, S.P. and Kaplan, N.O. eds.) Vol III, pp. 216-220, Academic Press, New York, N.Y.

Niehaus, W.G. & Hammerstedt, R.H. (1976) Mode of Orthophosphate Uptake and ATP Labeling by Mammalian Cells. Biochim. Biophys. Acta, 443, 515-524.

Orr, G.A. & Knowles, J.R. (1974) The Interaction of the Phosphonate Analogue of 3-Phospho-D-glycerate with Phosphoglycerate Kinase. Biochem. J. 141, 721-723.

O'Sullivan, W.J. & Perrin, D.D. (1964) The Stability Constants of Metal-Adenine Nucleotide Complexes. Biochemistry, 3, 18-26.

Parker, J.C. & Hoffman, J.F. (1967) The Role of Membrane Phosphoglycerate Kinase in the Control of Glycolytic Rate by Active Cation Transport in Human Red Blood Cells. J. Gen. Physiol. 50, 893-916.

Pegoraro, B. & Lee, C.-Y. (1978) Purification and Characterization of Two Isozymes of Phosphoglycerate Kinase from the Mouse. Biochim. Biophys. Acta, 522, 423-433.

Perrin, D.D. (1965) *Dissociation Constants of Organic Bases in Aqueous Solution*, pp. 408-409, Butterworths, London.

Philips, M., Roustan, C., Fattoum, A. & Pradel, L.-A. (1978) Yeast 3-Phosphoglycerate Kinase. Essential Arginyl Residues at the 3-Phosphoglycerate Binding Site. Biochim. Biophys. Acta, 523, 368-376.

Phipps, D.A. (1976) *Metals and Metabolism*, pp. 23-114, Oxford University Press, London.

Proverbio, F. & Hoffman, J.F. (1977) Membrane Compartmentalized ATP and Its Preferential Use by the Na,K-ATPase of Human Red Cell Ghosts. J. Gen. Physiol. 69, 605-632.

Rao, B.D.N., Cohn, M. & Scopes, R.K. (1978) ^{31}P NMR Study of Bound Reactants and Products of Yeast 3-Phosphoglycerate Kinase at Equilibrium and the Effect of Sulfate Ion. J. Biol. Chem. 253, 8056-8060.

Rao, D.R. & Oesper, P. (1961) Purification and Properties of Muscle Phosphoglycerate Kinase. Biochem. J. 81, 405-411.

Recktenwald, D. & Hess, B. (1977) Allosteric Influence of Anions on Mitochondrial ATPase of Yeast. FEBS Lett. 76, 25-28.

Reeves, R.E. & South, D.J. (1974) Phosphoglycerate Kinase (GTP). An Enzyme from *Entamoeba histolytica* Selective for Guanine Nucleotides. Biochem. Biophys. Res. Commun. 58, 1053-1057.

Ritter, H. & Schmitt, J. (1974) Interspecific Variability of Red Cell Phosphoglycerate Kinase in Primates. Humangenetik, 21, 93-96.

Rogers, K. & Weber, B.H. (1977) Evidence for an Essential Role for Arginyl Residues for Yeast Phosphoglycerate Kinase. Arch. Biochem. Biophys. 180, 19-25.

Ronquist, G. (1967) Fomation of Adenosine Triphosphate by a Membrane Fraction from Human Erythrocytes. Acta Chem. Scand. 21, 1484-1494.

Roustan, C., Brevet, A., Pradel, L.-A. & van Thoai, N. (1973) Yeast 3-Phosphoglycerate Kinase. Interaction of Enzyme with Substrates Studied by Partial Isotopic Exchange and Difference Spectrophotometry. Eur. J. Biochem. 37, 248-255.

Roustan, C., Fattoum, A. & Pradel, L.-A. (1976) Physicochemical and Kinetic Properties of Iodinated Yeast 3-Phosphoglycerate Kinase. Biochemistry, 15, 2172-2177.

Sarkar, B. (1977) Concept of Molecular Design in Relation to Metal-Binding Sites of Proteins and Enzymes. In: *Metal-Ligand Interactions in Organic Chemistry and Biochemistry* (Pullman, B. and Goldblum, N.,eds.) part 1, pp. 193-228, D. Reidel Publishing Company, Dordrecht-Holland.

Scatchard, G., Scheinberg, I.H. & Armstrong, S.H. (1950) Physical Chemistry of Protein Solutions. IV. The Combination of Human Serum Albumin with Chloride Ion. J. Am. Chem. Soc. 72, 535-540.

Schrier, S.L., Ben-Bassat, I., Junga, I., Seeger, M. & Grumet, F.C. (1975) Characterization of Erythrocyte Membrane-Associated Enzymes (Glyceraldehyde-3-Phosphate Dehydrogenase and Phosphoglyceric Kinase). J. Lab. Clin. Med. 85, 797-810.

Scopes, R.K. (1969) Crystalline 3-Phosphoglycerate Kinase from Skeletal Muscle. Biochem. J. 113, 551-554.

Scopes, R.K. (1971) An Improved Procedure for the Isolation of 3-Phosphoglycerate Kinase from Yeast. Biochem. J. 122, 89-92.

Scopes, R.K. (1973) 3-Phosphoglycerate Kinase. In: *The Enzymes* (Boyer, P.D. ed.) Vol. VIII, pp. 335-351, Academic Press, New York, N.Y.

Scopes, R.K. (1976) Activation and Inhibition of Phosphoglycerate Kinase by Anions. Abstr. 10th International Congress of Biochemistry, no. 07-4-136.

Scopes, R.K. (1977a) Purification of Glycolytic Enzymes by using Affinity-Elution Chromatography. Biochem. J. 161, 253-263.

Scopes, R.K. (1977b) Multiple Enzyme Purifications from Muscle Extracts by Using Affinity-Elution-Chromatographic Procedures. Biochem. J. 161, 265-277.

Scopes, R.K. (1978a) The Steady-State Kinetics of Yeast Phosphoglycerate Kinase. Anomalous Kinetic Plots and The Effects of Salts on Activity. Eur. J. Biochem. 85, 503-516.

Scopes, R.K. (1978b) Binding of Substrates and Other Anions to Yeast Phosphoglycerate Kinase. Eur. J. Biochem. 91, 119-129.

Segel, G.B. Feig, S.A., Glader, B.E., Müller, A., Dutcher, P. & Nathan, D.G. (1975) Energy Metabolism in Human Erythrocytes: The Role of Phosphoglycerate Kinase in Cation Transport. Blood, 46, 271-278.

Segel, I.H. (1975) *Enzyme Kinetics*, pp. 360-372, John Wiley and Sons, Inc., New York, N.Y.

Sigel, H., Becker, K. & McCormick, D.B. (1967) Ternary Complexes in Solution. Influence of 2,2'-Bipyridyl on the Stability of 1:1 Complexes of Co^{2+}, Ni^{2+}, Cu^{2+}, and Zn^{2+} with Hydrogen Phosphate, Adenosine 5'-Monophosphate and Adenosine 5'-Triphosphate. Biochim. Biophys. Acta, 148, 655-664.

Shows, T.B. & Brown, J.A. (1975) Human X-Linked Genes Regionally Mapped Utilizing X-Autosome Translocations and Somatic Cell Hybrids. Proc. Natl. Acad. Sci. USA 72, 2125-2129.

Sober, H.A. (ed.) (1968) *Handbook of Biochemistry*, pp. J51-J136, Chemical Rubber Company, Cleveland, Ohio.

Spragg, S.P., Wilcox, J.K., Roche, J.J. & Barnett, W.A. (1976) The Association of Yeast Phosphoglycerate Kinase (EC 2.7.2.3). Biochem. J. 153, 423-428.

Stewart, A.A. & Scopes, R.K. (1978) Phosphoglycerate Kinase B from Ram Testis. Purification, Characterisation and Comparison with the Muscle Isoenzyme. Eur. J. Biochem. 85, 89-95.

Stinson, R.A. (1974) Chromophoric Labeling of Yeast 3-Phosphoglycerate Kinase with an Organomercurial. Biochemistry, 13, 4523-4529.

Stinson, R.A. (1977) Use of Isoelectric Focusing and a Chromophoric Organomercurial to Monitor Urea-Induced Conformational Changes of Yeast Phosphoglycerate Kinase. Biochem. J. 167, 65-70.

Suzuki, K., Eguchi, C. & Imahori, K. (1977) Affinity Labeling of Adenine Nucleotide-Related Enzymes with Reactive Adenine Nucleotide Analogs II. Affinity Labeling of Phosphoglycerate Kinase with a Reactive AMP Analog. J. Biochem. (Tokyo) 81, 1393-1399.

Szabolsci, G., Bisku, E. & Szörényi, E. (1959) Comparative Studies on D-Glyceraldehyde-3-Phosphate Dehydrogenase VII. Studies on the Digestibility of the Enzyme Isolated from Various Mammals. Biochim. Biophys. Acta, 35, 237-241.

Szabolsci, G. & Bisku, E. (1961) Structural Changes Induced by the Blocking of Protein SH Groups. Biochim. Biophys. Acta, 48, 335-341.

Tanabe, T., Wada, K., Ogiwara, H. & Numa, S. (1977) Effects of Allosteric Regulators on Proteolysis of Rat Liver Acetyl Coenzyme A Carboxylase by Lysosomal Extract. FEBS Lett. 82, 85-88.

Tang, C.C., Davalian, D., Huang, P. & Breslow, R. (1978) Models of Metal Binding Sites in Zinc Enzymes. Synthesis of Tris[4(5)-imidazolyl]carbinol(4-TIC), Tris (2-imidazolyl)carbinol(2-TIC), and Related Ligands, and Studies on Metal Complex Binding Constants and Spectra. J. Am. Chem. Soc. 100, 3918-3922.

Tanswell, P., Westhead, E.W. & Williams, R.J.P. (1974) Inhibition of Yeast Phosphoglycerate Kinase by Lanthanide-ATP Complexes. FEBS Lett. 48, 60-63.

Tanswell, P., Westhead, E.W. & Williams, R.J.P. (1976) Nuclear-Magnetic-Resonance Study of the Active-Site Structure of Yeast Phosphoglycerate Kinase. Eur. J. Biochem. 63, 249-262.

Tillmann, W., Cordua, A. & Schröter, W. (1975) Organization of Enzymes of Glycolysis and of Glutathione Metabolism in Human Red Cell Membranes. Biochim. Biophys. Acta, 382, 157-171.

Torchinskii, Y.M. (1974) *Sulfhydryl and Disulfide Groups of Proteins*, pp. 40-138, Plenum Publishing Corporation, New York, N.Y.

Vande Berg, J.L., Cooper, D.W. & Close, P.J. (1973) Mammalian Testis Phosphoglycerate Kinase. Nature, 243, 48-49.

Vande Berg, J.L., Cooper, D.W. & Close, P.J. (1976) Testis Specific Phosphoglycerate Kinase B in Mouse. J. Exp. Zool. 198, 231-240.

Walsh, C.T. & Spector L.B. (1971) A Phosphoenzyme Intermediary in Phosphoglycerate Kinase Action. J. Biol. Chem. 246, 1255-1261.

Watson, H.C., Bryant, T.N., Walker, N.P.C., Shaw, P.J. & Wendell, P.L. (1977) The Active Site Structure of Yeast Phosphoglycerate Kinase. Biochem. Soc. Trans. 5, 652-654.

Weber, K. & Osborn, M. (1969) The Reliability of Molecular Weight Determinations by Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis. J. Biol. Chem. 244, 4406-4412.

Wendell, P.L., Bryant, T.N. & Watson, H.C. (1972) Low Resolution Structure of Yeast Phosphoglycerate Kinase. Nature, 240, 134-136.

Whitehead, E.P. (1978) Co-operativity and the Methods of Plotting Binding and Steady-State Kinetic Data. Biochem. J. 171, 501-504.

Yap, W.T. & Saroff, H.A. (1971) Binding of Na and H Ions to 2,3-Diphosphoglycerate. Abstr. Meet. Am. Chem. Soc. 162, no. 157.

Yoshida, A. (1972) Micro Method for Determination of Blocked NH_2-Terminal Amino Acids of Protein: Application to Identification of Acetylserine of Phosphoglycerate Kinase and Pyroglutamic Acid of Glucose 6-Phosphate Dehydrogenase. Anal. Biochem. 49, 320-325.

Yoshida, A. & Watanabe, S. (1972) Human Phosphoglycerate Kinase I. Crystallization and Characterization of Normal Enzyme. J. Biol. Chem. 247, 440-445.

Yoshida, A., Watanabe, S., Chen, S.-H., Giblett, E.R. & Malcolm L.A. (1972) Human Phosphoglycerate Kinase. II Structure of a Variant Enzyme. J. Biol. Chem. 247, 446-449.

APPENDIX I

PUBLISHED PAPERS ARISING FROM THIS WORK

Wrobel, J.A. & Stinson, R.A. (1978) Anion Binding to Yeast Phosphoglycerate Kinase. Eur. J. Biochem. 85, 345-350.

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